Ion Channels in Drug Discovery
- focus on biological assays

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- focus on biological assays

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Front page (a) Side view of the GABA-A model; the trans membrane domain (TMD) (bottom) spans through the lipid membrane (32 Å) and partly on the extracellular side (12 Å), while the ligand binding domain (LBD) (top) is completely extracellular (60 Å). (b) The LBD viewed from the extracellular side (diameter of 80 Å). (c) The TMD viewed from the extracellular side. Arrows and ribbons (which correspond to β-strands and α-helices, respectively) are coloured according to position in each individual subunit sequence, as well as in the TM4 of each subunit which is not bonded to the rest of the model (with permission from Campagna-Slater and Weaver, 2007 Copyright © 2006 Elsevier Inc.).

Published by Karolinska Institutet.

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REPROPRINT AB
Stockholm 2008
www.reproprint.se
Gårdsvägen 4, 169 70 Solna
Denne afhandling er tilegnet min elskede Tine som fulgte med mig rundt i verden for at jeg kunne forfolge mine drømme.

“In The Middle Of Difficulty Lies Opportunity”
Albert Einstein
ABSTRACT

Ion channels are well characterised drug targets. However, the techniques used to study ion channel pharmacology have not been particularly applicable to modern drug discovery. The aim of this thesis was to examine the usefulness of both established and novel methods used in the discovery and pharmacological characterisation of drugs interacting with ion channels. Three different classes of ion channels have been studied; the GABA<sub>A</sub> receptor representing the classical ligand-gated ion channel, the voltage-gated sodium channel (Nav-1.7) and the newly discovered non-selective cation channel, Transient Receptor Potential MELASTATIN-8 (TRPM8).

Due to the difficulty in expression and purification of membrane proteins, no crystal structures of GABA<sub>A</sub> receptors are currently available and structure-based drug design is not directly applicable. Using pharmacophore modelling and site-directed mutagenesis, ligand interactions were studied at a structural level. A pharmacophore model for the benzodiazepine binding site (BzR) on the GABA<sub>A</sub> receptor, based on structure-activity relationship studies for 136 different ligands from 10 structurally different classes was used to model flavonoids binding to the GABA<sub>A</sub> receptor. By synthesising a series of flavonoids, the structure activity relationship (SAR) was investigated using <sup>3</sup>H-flumazenil binding to rat cortical membranes in vitro. The results demonstrate that flavonoids with high affinity for the BzR spanning the whole efficacy range from agonists to inverse agonists can be synthesised and the receptor binding properties of the flavonoids can successfully be rationalised in terms of a comprehensive pharmacophore model.

In order to investigate which arginine residues potentially contribute to the formation of the GABA binding pocket, six arginines conserved in all human GABA<sub>A</sub> receptor α subunits as well as two non-conserved arginines in the extracellular, N-terminal segment of the α5 subunit, were substituted by lysines. The individual α5 subunit mutants were co-expressed with human β2 and γ2s GABA<sub>A</sub> receptor subunits in CHO cells using transient transfection. Electrophysiological whole-cell patch-clamp recordings showed that, of the eight arginine residues tested, only the two arginines, at position 70 and 123, appear to be essential for GABA-gated chloride current, since the EC<sub>50</sub> values of the two mutant constructs increase more than 100-fold as compared to wild-type α5β2γ2s GABA<sub>A</sub> receptors.

Ion channels are challenging targets particularly in the early phases of the drug discovery process, due to the lack of technologies available to screen large numbers of compounds in functionally relevant assays. The human sodium channel Nav-1.7 was stably expressed in HEK293 cells and 3 high throughput screening (HTS) assays were compared. 1) A Li<sup>+</sup> flux atomic absorption spectroscopy (AAS) assay, 2) a Fluorometric Imaging Plate Reader (FLIPR) membrane potential assay 3) a Fluorescent Energy Transfer (FRET) based membrane potential assay. These 3 assays were then compared to an automated electrophysiological assay (the Ionworks-HT platform) to characterize eleven known sodium channel inhibitors.
The results demonstrated that all 3 HTS assays are suitable for the identification of NaV-1.7 inhibitors, but for an HTS assay the Li⁺-flux assay was more robust than the FLIPR and FRET based membrane potential assays. Furthermore, there was a better correlation between the Ionworks assay and the Li⁺-flux assay regarding the IC₅₀ values of the sodium channel inhibitors investigated.

Human TRPM8 channels stably expressed in HEK293 were used to develop a FLIPR based HTS assay using the calcium sensitive dye Fluo-4. The two known TRPM8 agonists menthol and icilin induced [Ca²⁺]ᵢ increases with EC₅₀ values comparable to electrophysiological measurements. Screening of a compound library identified 15 novel antagonists, which were characterized using both the FLIPR assay and two-electrode voltage clamp electrophysiology in Xenopus oocytes expressing TRPM8. The antagonists were additionally tested in a FLIPR assay using cold as the agonist, which additionally demonstrated concentration dependent antagonism. These results show that menthol, icilin and cold can all be used as agonists when searching for antagonists and demonstrate that a cellular HTS assay for investigating the pharmacological activity of TRPM8 ligands can be developed.

The work in this thesis illustrates the validity of multiple methods and technologies when studying ion channel pharmacology. Traditional ligand binding assays in combination with molecular modelling are important tools for structure activity relationship studies, but functional assays are needed to determine ligand efficacy. The functional cellular assays that have become available recently to study ion channels have shown great potential in terms of screening throughput and delivering quality data. These assays are capable of determine both the pharmacological properties of ion channel ligands and perform SAR studies. With these developments ion channels may become more tractable targets for the pharmaceutical industry, leading to new improved drugs for the patients.

**Key words:** ion channels, GABAₐ receptor, sodium channels, TRPM8, drug discovery, high throughput screening assays, FLIPR, automated electrophysiology.
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   binding to the benzodiazepine site of the rat brain GABA-A receptor complex.

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    GABA-A receptor alpha-5 subunit are crucial for receptor function.
    Journal of Neurochemistry, 2000, 75, 1746-1753.

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     characterization of NaV-1.7 modulators.

IV. Dekermendjian, K., Dabrowski, M., Fredholm, B.B. and Larsson, O.
    Development of a high throughput FLIPR assay to study the pharmacological
    properties of TRPM8 ligands.
    Submitted to British Journal of Pharmacology.

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benzodiazepine receptor ligand with antagonistic properties on rat brain and human
recombinant GABA-A receptors in vitro.

M. Arginine residue 120 of the human GABA-A receptor alpha 1, subunit is essential
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<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
</tr>
<tr>
<td>AMPA</td>
<td>Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>BzR</td>
<td>Benzodiazepine receptor (binding site)</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>Intracellular calcium concentrations</td>
</tr>
<tr>
<td>$\text{Ca}_V$</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CD</td>
<td>Candidate drug</td>
</tr>
<tr>
<td>Cl$_V$</td>
<td>Voltage-gated chloride channel</td>
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<tr>
<td>Cys-loop</td>
<td>Cystein-loop</td>
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<td>DMPK</td>
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<td>DRG</td>
<td>Dorsal root ganglion</td>
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<tr>
<td>FLIPR</td>
<td>Fluorometric Imaging Plate Reader</td>
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<tr>
<td>FRET</td>
<td>Fluorescent energy transfer</td>
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<tr>
<td>GABA</td>
<td>$\gamma$-amino-butyric acid</td>
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<tr>
<td>GlyR</td>
<td>Glycine receptor</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<tr>
<td>HTS</td>
<td>High throughput screening</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational new drug</td>
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<tr>
<td>$\text{K}_V$</td>
<td>Voltage-gated potassium channel</td>
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<tr>
<td>nAChR</td>
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<td>NCE</td>
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<td>P2XR</td>
<td>Purinergic 2X receptor</td>
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<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
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<tr>
<td>TEVC</td>
<td>Two-electrode voltage clamp</td>
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<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
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<td>TRPM8</td>
<td>Transient receptor potential melastatin-8</td>
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<td>TRPV1</td>
<td>Transient receptor potential vanilloid receptor type-1</td>
</tr>
<tr>
<td>VIPR</td>
<td>Voltage-sensitive ion channel reader</td>
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<td>WT</td>
<td>Wild type</td>
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1 INTRODUCTION

The process of drug discovery is a long, complex, costly and highly risky endeavour involving a great number of scientists from various disciplines. In this process the pharmaceutical industry generally follows a relatively simple set of guidelines;

1) Establish a biological hypothesis of the pathophysiological mechanism involved in the disease of interest.
2) Identify and select the biological target (usually a protein) that is central for the treatment of the disease.
3) Develop a biological high throughput-screening (HTS) assay that can identify active compounds on the selected target.
4) Test $10^4$-$10^6$ of chemical substances from a compound library in the HTS assay.
5) Apply medicinal chemistry to the active compounds and establish lead series with known SAR (structure activity relationship).
6) Optimize the pharmacokinetic properties of the compounds for animal testing.
7) Test the compounds in animal models of the disease.
8) Establish toxicity test to make sure the compound is safe for testing on humans.
9) Start clinical trials, to demonstrate that the compound is effective and safe as a new medication.
10) Get the drug approved by regulatory authorities and market the drug.

This thesis focuses on steps 2-5 of this process and describes various biological assays developed to study the pharmacology of ion channels. The functional cellular assays that have become available recently to study ion channels show great potential in terms of screening throughput and delivering quality data to determine both the pharmacological properties of ion channel ligands and perform SAR studies. With these developments, ion channels may become more tractable targets for the pharmaceutical industry.

1.1 THE DRUG DISCOVERY PROCESS

Drug discovery and development is - simply put - the process whereby compounds with a specific pharmacological activity towards a specified biological target or function are identified, evaluated, and optimized for clinical applications. This may appear simple, but it is an extremely complex procedure. The cost of discovering and developing a new drug or new chemical entity (NCE) and launching it on the market has been estimated to 1000 million US$, and requires 12-14 years (DiMasi et al., 2003; Dickson and Gafnon, 2004). Furthermore, only 30% of drugs on the marked generate revenues that meet or exceeds average research and development cost, and 70% of total revenues are generated by only 20% of the products.

The main reason for this enormously high cost for developing new drugs is that most of the NCEs fail during the drug discovery process due to unsatisfactory pharmacological properties, unexpected toxicity, failure to achieve efficacy in clinical trials, or issues related to intellectual property (Kola and Landis 2004). With such a
tremendous investment to develop NCEs, it is not surprising that the pharmaceutical industry is constantly thinking in terms of effectiveness and trying to develop a streamlined process that could deliver NCEs faster and with less cost (for general information on drug discovery http://www.nature.com/nrd/index.html).

All companies therefore have documents describing the guidelines in the process and the requirements that have to be fulfilled to proceed with a project from one phase to the next. These guidelines and requirements are slightly different from one company to another, but essentially they will follow a scheme like the one exemplified below (Figure 1).

The Drug Discovery Process
as it looks in a typical major pharmaceutical industry

**Figure 1.** Target = Biological entity usually a protein. Hit = A primary active compound with specific binding properties, exceeding a certain threshold value in a given assay (ex. >50% inhibition of target activity or >50% potentiation on target activity). Validated/confirmed hit = the physiochemical properties of the initial hit is confirmed and a newly synthesized solid dry compound is made for conformation in concentration response experiments. SAR = Structure activity relationship, the consistent correlation of structural features or groups in the compounds with the biological activity in a given assay. Lead = A distinct chemical structure or series of structures demonstrating activity and selectivity in a relevant biological/pharmacological screen. This forms the basis for a directed medicinal chemistry effort for lead optimization and development with the purpose of identifying the best compound to be tried in the clinic (candidate drug = CD).

In this scheme, the process has been divided into two major phases, the preclinical phase (up to IND approval) and the clinical phase. This division between the preclinical and clinical phases of a drug discovery project is based on practical considerations since these phases of drug discovery typically are handled by two different
organizations (discovery and development) within a company. In figure 1, the arrows on top illustrate major decision points (milestones) in the process such as:
- Selecting the target.
- Selecting the chemical series to optimize for animal testing.
- Selecting the best leads to start clinical tests (candidate drug).
- IND application that has to be approved by regulatory authorities before clinical tests can begin.

At this point the preclinical phase is over and the project is transferred from the discovery organization over to the development organization. As illustrated above, the clinical phase of the whole process is by far the longest (and most expensive), however, this thesis will only focus on the very early part of the discovery process.

As illustrated in Figure 1, the initial step in drug discovery involves the identification and validation of a target, generally a protein, for which the pharmacological modulation of its activity is predicted to produce a desired effect. In drug discovery, the term “drugable target or drugability” is used to describe the proteins that a small molecule (drug) can affect, and these families of proteins include enzymes, G-protein coupled receptors (GPCRs), nuclear hormone receptors and ion channels. Currently prescribed drugs act on very few protein families (see Figure 2), and it has been suggested that the number of drugable targets is limited to ~300 proteins, compared to the total number of >30,000 different proteins in the human body (for review see Landry and Gies 2008).

![Current drug targets](image)

**Figure 2.** Marketed small-molecule drug targets by biochemical class (Modified from Hopkins and Groom 2002).

Target identification and validation may involve gene/protein expression profiling, phenotype analysis in cell culture and transgenic mouse models, and evaluation of humans with gene deletion/mutation (Knowles and Gromo, 2003). The initial identification of compounds interacting with the target usually involves high-throughput screening (HTS) of diverse small molecule collections or structurally selected compounds with known or theoretically predicted activity against a target
(Walters and Namchuk, 2003; Bleicher et al., 2003). Initial drug discovery thus requires a robust biological assay of target activity and a collection of compounds for testing. "Hits" from initial screening are evaluated on the basis of many criteria, including potency, specificity, toxicity, pharmacology, biopharmaceutical properties, and efficacy in animal models, to select lead compounds for optimization by synthetic chemistry and more extensive preclinical evaluation in animal models (Kenakin, 2003). These preclinical data form the basis of the preclinical pharmacology section of an IND application to carry out clinical trials.

1.1.1 Assay development

After the biological target has been identified, the development and selection of a screening assay can begin, and this is one very important aspect of the drug discovery process (Walters and Namchuk, 2003). Generally the screening assay can be seen as the first filter in selecting a potential lead compound (compounds to develop further) from the total company collection (typically ~1-5 million) of compounds. The screening assay should be designed to assay target activity with high sensitivity and specificity in a setting that reflects the behaviour of the target in-vivo. This is difficult to accomplish, thus, selecting a screening assay is always a compromise between throughput (how many compounds can be screened) and the biological/pharmacological validity of the data obtained (for review see Pereira and Williams 2007).

Overall, assays can be divided in non-functional or functional assays, based on whether the function/activity of the target can be monitored by the assay or not. The initial choice of screening assay is highly dependent on what type of biological target the project is focusing. The identification of inhibitors of a purified enzyme having a specific function and using a simple colorimetric readout is conceptually much more straightforward and easier to implement than a complex cell-based assay involving the expression of proteins in a host cell line. However, the cell-based assay potentially could deliver more biologically relevant data (Swinney, 2004; Walters and Namchuk, 2003).

For GPCRs and ion channels, the simplest assays are radio-ligand competition binding assays, measuring the ability of the test compounds to displace the radio-labelled high affinity ligand from the binding site on the protein. These binding assays generate very reliable and robust data, however being non-functional assays, they give no information on the agonistic or antagonistic characteristics of the compounds tested. As a consequence, other assays have to be developed to determine the in-vitro pharmacological effect, and thus require additional resources for follow-up evaluation of active compounds identified in a primary screen (Luiz and Kenakin 1999).

The best functional cell-based assay for ion channel activity are electrophysiological measurements using the patch-clamp method. This method provide very detailed information of the interaction and pharmacology of the compounds binding to the target, but this technique has (until very recently) suffered from extremely low throughput (1-3 compounds can be tested/day), as compared to the binding assays ($10^3$-$10^6$ compounds/day) (Bleicher et al., 2003).
Another important consideration when choosing the primary screening assay is that the assay of choice will determine the type of active compounds that eventually will be found. Due to the nature of the binding assay, mainly compounds binding within (or allosterically modulating) the same pocket as the labelled ligand will be determined as actives, while a functional assay will identify active compounds independent on the binding site, as long as the compound modulate the function of the protein. Therefore, functional assays are more likely to identify actives having a novel mechanism of action (ex. allosteric modulators) as compared to binding assays, which often identify competitive antagonists/agonists (Swinney, 2004).

The requirements of a screening assay include: good target sensitivity and specificity, robust readout, day-to-day reproducibility, technical simplicity, suitability for automation, and preferably low cost. The technical aspects and cost of a screening assay are particularly important if the assay is to be repeated on hundreds of thousands of test compounds by utilizing HTS technology. The cost per single assay-point of an optical readout of activity in a cell-free system can be as low as US$ 0.10-0.50, whereas the cost of a single assay-point, in a complex cell-based assay can exceed US$ 5.00-10.00 (Birch et al., 2004). A complex cell-based assay may require multiple reagent additions and liquid exchange during hours/days, mechanical steps such as filtration, and a kinetic multi-time-point readout. Assays that are amenable to optical readout (absorbance, fluorescence, luminescence) are usually more robust, easily automated, and less expensive than assays utilizing radioisotopes or electrophysiology (Walters and Namchuk, 2003).

1.1.2 High throughput screening

The implementation of a screening assay for HTS often requires automation due to the repetitive and labour-intensive nature of the screening process. HTS is generally performed using standard 96- or 384-well plastic plates and typically, 1,000-10,000 individual assay points are generated daily in HTS mode (10,000 data-points using 384-well plates imply ~30 plates have to be screened). Several types of robotic instruments for automated compound screening are available commercially, one example from AstraZeneca is shown in the picture below.

This instrument utilizes a robotic arm mounted on a trail with the workstations on each side where multiwell plates and pipette tips can be manipulated. Multiwell plates containing cultured cells are loaded onto a carousel in a CO₂ incubator with a rear entrance for robot access. A carousel (carousel-A) on the table surface is loaded with plates containing test compounds and/or containers containing pipette tips and multiwell plates. The workstations shown in the photograph include a bar code reader, a station to remove plastic lids of multiwell plates, cell wash station, liquid handling workstation, the Fluorometric Imaging Plate Reader (FLIPR) instrument, equipped with dual syringe pumps for solution additions during the assay. The liquid handling workstation accesses pipetting tools to add, remove, or transfer compounds/solutions from well to well or plate to plate.
The robotic system is programmed to carry out complex operations over many hours and, once set up, requires little attention during a run. The great advantage with a system like the one shown here is that it can be tailor-made to the application needed. The one shown here is setup for real time fluorescence measurements on the FLIPR, but other instruments could easily be integrated in the system.

Example of an automated robotic system for HTS

The data processing capabilities are important to the screening process, since multi-time-point assays can produce millions of readout points per day. Commercial bioinformatics software is available to capture information in relational databases for hypothesis testing and compound optimization. Various statistical parameters have been applied to evaluate assay sensitivity and specificity. A widely used statistical parameter is the Z’ factor, which provides a measure of the reliability of hit selection based on the dynamic range and intrinsic variability of the assay (Zhang et al., 1999).

1.1.3 Hit selection

Although compound screening can be challenging and expensive, the primary HTS is generally completed in less then two months, depending on the number of compounds to be tested and the complexity of the screen. The secondary evaluation of hits from a HTS is a more time-consuming process, the goal of which is to select lead compounds for further development. The first step in the hit selection is to define a “Hit”, by determining the activity of the compounds (cut-off value ex. >50% inhibition of target activity).

Considering that e.g. 600,000 compounds have been screened in an HTS campaign in a 30μM single point concentration, the hit-rate has a tremendous impact on the number of compounds that have to be retested (Walters and Namchuk, 2003). Typically, a HTS campaign has a 0.1-1% initial hit-rate, which could give 600-6000
initial hits. Initial evaluation of hits is designed to identify false positives. Retesting of hits with the original HTS assay is done to confirm activity, followed by target-specific confirmation. For example, in a screen involving transfected cells expressing the target of interest, lack of activity could be confirmed in wild type cells. More definitive and time-consuming "secondary screening" assays are performed to confirm target specificity. For example, electrophysiological analysis of a target ion channel can be done after hits are identified from a primary screening involving ion-sensitive fluorescent dyes or radioisotopes.

It is preferable that the technology of the primary and secondary assays is different, thereby avoiding methodological shortcomings responsible for any hit selection (for general methods in drug discovery see Lutz and Kenakin, 1999). The structures of confirmed hits are examined to search for common motifs and for initial evaluation of potential medicinal value, which often includes computational "in silico" modeling and determination of physicochemical properties like solubility and pKa. Prioritization of confirmed hits is done by evaluation of compound potency (concentration-response measurements) and chemical structure of each confirmed hit.

1.2 ION CHANNELS

Currently, approximately 300 unique human ion channel genes have been identified by the human genome project (Venter, et al., 2001) and, although the functional significance for a majority of these ion channels is unknown, this target class is very appealing to the pharmaceutical industry since these proteins are key components in a vast variety of biological processes and are present in most cells. Although the drugs launched targeting ion channels are successful in the cardiovascular and CNS disease area they only act upon a handful of ion channels like GABA<sub>A</sub>, Ca<sub>V</sub>, Na<sub>V</sub>, 5-HT<sub>3</sub> and K<sub>V</sub> (Hopkins and Groom 2002).

Ion channels are pore forming membrane-spanning proteins through which specific inorganic ions such as Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Cl<sup>-</sup> can flow down the electrochemical gradient at a rate of up to 10<sup>8</sup> ions/sec, inducing large changes in the membrane potential as well as affecting a variety of signalling pathways (Armstrong and Hille 1998). Most ion channels are selective for a given type of ion, therefore the ion channels have been classified according to the ions they are permeable to i.e. sodium channels (Na<sub>V</sub>), potassium channels (K<sub>V</sub>), calcium channels (Ca<sub>V</sub>), chloride channels (Cl<sub>V</sub>) or non-selective cation channels (Hille 1992). Ion channels are further divided into two broad categories according to their activation mechanism namely ligand-gated or voltage-gated ion channels.

The ligand-gated channels require a molecule, physiologically often the neurotransmitter, to bind to the channel thereby changing the conformation of the protein, which in turn opens the channel allowing the specific ion to pass. Examples of classical ligand-gated channels (and their agonists) are GABA<sub>A</sub> (GABA), GlyR (glycine), 5HT<sub>3</sub> (serotonin), nAChR (acetylcholine), NMDA, AMPA and Kainate (glutamate), P<sub>2X</sub>R (ATP) (for general information and references on ligand-gated ion channels see the “Ligand-gated ion channel database http://www.ebi.ac.uk/compneur-
Within the last few years a new family of ligand-gated ion channels the Transient Receptor Potential (TRP) channel family have emerged. This family of ion channels is activated by various neuromodulators and physical stimuli such as heat, cold, protons and force (Clapham, 2003; Venkatachalam and Montell 2007). Most of these ligand-gated ion channels exist as heteromers composed of several subunits leading to a vast diversity of subtypes within each channel class.

**Figure 3.** Structure of ligand-gated ion channels. Alpha subunits of the three families of ligand-gated ion channels are illustrated (modified from Landry and Gies 2008).

Ligand-gated ion channels display a very diverse array of topologies both in the number of transmembrane (TM) spanning regions (from 2-6, see Figure 3-4 for examples) as well as a large variation in the C- and N-terminal regions. Typically, within each subfamily multiple subunit types comprise the functional channel, illustrated by the GABA<sub>A</sub> receptor, assembled as a pentamer of three different subunits (discussed in more detail later).

Voltage-gated channels are not dependent on a ligand for activation, but are activated by changes in the membrane potential with the most common voltage gated channels being Na<sub>V</sub> channels, K<sub>V</sub> channels, Ca<sub>V</sub> and Cl<sub>V</sub> channels (for an overview of voltage-gated channels see Armstrong and Hille 1998). In contrast to ligand-gated channels voltage-gated ion channels follow a more uniform topology in which four domains, each consisting of a six TM spanning region, build up the functional ion channel complex (Figure 4 and 6 for examples).

Each α-subunit contains intracellular N- and C-termini and is believed to have six hydrophobic transmembrane segments (S1–S6) that are linked by short extracellular and intracellular loops (figure 4a). The transmembrane segment S4 contains a large number of positively-charged amino acids and is believed to function as the voltage-sensor. The so-called P-loop, which connects S5 and S6, re-enters but does not traverse the membrane and contains amino acid residues that determine the permeability properties of the channel.
Voltage-gated $\mathrm{Na}^+$ channel ($\mathrm{Na}_V\alpha$-subunits) and $\mathrm{Ca}^{2+}$ channel ($\mathrm{Ca}_V\alpha$1-subunits) display similar overall membrane topology and contain similar structural elements. In contrast, $\mathrm{Na}_V$ and $\mathrm{Ca}_V$ subunits are composed of four homologous domains (see figure 6 illustrating a $\mathrm{Na}^+$ channel), each of which corresponds to one of the four $\mathrm{K}_V\alpha$-subunits that form a functional $\mathrm{K}^+$ channel. In figure 4 the illustration on the right shows the proposed circular arrangement of four $\mathrm{K}_V\alpha$-subunits with the ion permeable pore located at the center (Catterall et al., 2005). Also, this illustration represents the arrangement of the four homologous domains of a single $\mathrm{Na}_V\alpha$-subunit or $\mathrm{Ca}_V\alpha$1-subunit (Figure 4B).

![Diagram of membrane topology of a voltage-gated ion channel](image)

**Figure 4.** Proposed membrane topology of the pore-forming subunit of a voltage-gated ion channel (and the TRP channels have comparable topology). **A** The illustration on the left shows the proposed membrane topology of the archetypal voltage-gated $\mathrm{K}^+$ channel ($\mathrm{K}_V\alpha$-subunit). **B** Four subunits must co-assemble to form a functional channel (from McGivern 2007).

When an ion channel opens, permeant ions will flow passively in a direction that is determined by the electrochemical forces acting on the ions. The magnitude and time course of ion flow (current) is determined by the electrochemical gradient, the number of open channels and the kinetics of gating (Hille 1992). The flow of ions across cell membranes essentially provides the energy required for initiation of these downstream signaling events. Active ATP-utilizing pumps are responsible for maintaining the ionic concentration gradients that are necessary for ion channels to perform their functions.

### 1.2.1 Ion channels and pain

Ion channels play a major role in many physiological processes, and in neuronal communication they are vital. Neurons receive electrical input signals, integrate these signals and transmit the information to target cells via transmitter release and ion...
channels are central in this intra- and inter-cell communication. The function of sodium channels in the firing of action potential and the propagation of the electrical signal from the nerve cell body down the axon to the synapse is well established. Likewise it is well accepted that the majority of all fast synaptic activity in the CNS is mediated through ion channels as a balance between the main excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA (Hille 1992).

Pain arises as a consequence of electrical impulses generated in response to painful stimuli, which can be mechanical, chemical or thermal. These impulses travel from nociceptive neurons in the dorsal root ganglion (DRG) originating in the periphery of the body, through the spinal cord to the brain (see Hunt and Mantyh 2001 for review). Despite the fact that acute nociceptive pain functions as a biological warning sign, chronic pain is a huge medical problem. Chronic pain is persistent and classified as inflammatory or neuropathic pain. Inflammatory pain is caused by injury, irritation or infection of peripheral tissue, while neuropathic pain is caused by damage of neurons in the CNS. Patients with chronic pain often suffer from hypersensitivity to chemical, mechanical and/or thermal stimulation (hyperalgesia), aggravated painful response to normally painful stimuli or allodynia (painful response to normal stimuli) (Woolf 2004). Since the etiologies and mechanisms of these various forms of pain are different, the identification of the molecular entities of the nociceptor (receptors in the nociceptive neurons) has been an area of intensive research (Scholz and Woolf 2002).

The molecular diversity of ion channels in the nociceptive neurons has been discovered within the last 15 years and includes sodium channels, calcium channels and TRP channels (reviewed by Scholz and Woolf, 2002). These channels give rise to the nociceptive impulse sensed as pain in the brain, and blocking or modulating these channels is a major goal for pharmaceutical treatment of pain today. Changes in the activity or these channels are likely to underlie the symptoms seen in chronic as well as neuropathic pain. Given the diversity of ion channel subtypes, one major goal for the pharmaceutical industry is to identify small molecules interacting specifically with only one subtype of a given ion channel, thereby reducing the risk of unwanted side effects (Birch et al., 2004). This goal has traditionally been hampered by the methodologies available to investigate ion channel pharmacology, but recently novel technologies to study ion channels have emerged and this will be discussed later in this thesis.

1.2.2 The GABA$_A$ receptor

In the mammalian CNS inhibitory neurotransmission is mainly mediated by the neurotransmitter $\gamma$-aminobutyric acid (GABA). Since one third of all synapses in the CNS is estimated to be GABAergic, modulating this system have important physiological consequences. Clinically important pharmaceuticals such as benzodiazepines, anaesthetics, barbiturates and neuro-steroids, all act through the GABA$_A$ receptor. These drugs produce a variety of behavioural effects ranging from hypnotic, sedative, anticonvulsant, muscle relaxant, and anxiolytic to convulsant, memory enhancement and anxiogenic, making the GABA$_A$ receptor an important drug target (Sieghart 2006).
GABA exerts its physiological effects by binding to two different receptor types in the postsynaptic neuronal membrane, the GABA_A and GABA_B receptors. The GABA_B receptor belongs to the G-protein coupled receptor superfamily, while the GABA_A receptors are ligand-gated chloride ion channel complexes (for review see Sieghart 2006). GABA mediates its fast inhibitory effects by activating the GABA_A receptor, inducing a conformational change opening the chloride ion-channel, permitting chloride ions to flow into the cell, thus hyperpolarising the neuron. The GABA_A receptor is a member of the cystein-loop (Cys-loop) ligand-gated ion-channel (LGIC) family, which includes the nicotinic acetylcholine, 5-HT_3, glycine and GABA_A receptor. This grouping into a LGIC family is based on the high degree of homology between these proteins.

A common structural feature for these ion channels is that they contain a large extracellular N-terminal with a conserved cys-loop and four transmembrane regions (TM I, II, III and IV) with a large intracellular loop between TM III and TM IV (see figure 3). The majority of structural information from these LGIC has come from the nicotinic acetylcholine receptor since it has been possible to resolve its structure at a resolution of 4.6 Å, while such detailed structural information have not been available from other of the LGIC family members. Based on the homology with the nicotinic acetylcholine receptor, the GABA_A receptors are thought to form heteropentameric chloride ion channels (Figure 5).

**Figure 5.** Proposed topography of the GABA_A receptor. Left, a cross-section in the plane of the membrane. Right, putative binding sites for allosteric ligands (modified from Landry and Gies 2008).

The GABA_A receptor gene family consist of six α, four β, three γ, one δ, one ε, one σ and three ρ subunits, each encoded by different genes. These receptor subunits are heterogeneously expressed throughout the CNS, with 7-10 major GABA_A receptor subtypes thought to exist in the brain, suggesting that different GABA_A receptor subtypes have different physiological functions (see Sieghart 2006 for review). A majority of GABA_A receptors are composed of a α-β-γ subunit combinations, while the GABA_C receptors (classified as a subtype of the GABA_A receptors) are homopentamers composed of ρ subunits. In addition to GABA binding sites, the GABA_A receptor chloride channel complex possesses binding sites for compounds that allosterically modify the chloride channel gating of GABA, such as benzodiazepines, β-carbolines, barbiturates and neurosteroids (figure 5).
Pharmacological effects of benzodiazepines (anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic) make them the most important GABA\(_A\) receptor modulating drugs in clinical use (Möhler et al., 2002). Benzodiazepines do not interact directly with the GABA binding sites, but exert their action by binding to allosteric sites within the GABA\(_A\) receptor complex, thereby modulating the properties of the GABA binding sites changing the GABA-induced neuronal membrane conductance for chloride ions, resulting in membrane hyperpolarisation, leading to a reduction in neuronal excitability. Compounds acting on benzodiazepine binding sites which allosterically modulate the chloride channel gating properties of GABA are traditionally termed “agonists”, and “inverse agonists”, while a third group of compounds “antagonists” have no effect on the chloride channel per se but exert their action by blocking the effects of agonists and inverse agonists.

It has to be stressed that benzodiazepines and related compounds per se are not agonists or inverse agonists at GABA\(_A\) receptors. GABA is the agonist at these receptors and benzodiazepines modulate the agonistic action of GABA by allosteric interaction. That is why the terms “positive allosteric modulators”, “negative allosteric modulators” and “neutralising allosteric modulators” for “agonists”, “inverse agonists” and “antagonists”, respectively, have been suggested. Both partial positive and partial negative allosteric modulators exist. There is evidence that these partial agonists (partial positive allosteric modulators) may not produce the physical dependence apparent with the full agonists (full positive allosteric modulators) and thus be suitable lead substances for the development of new anxiolytics. The side effects of benzodiazepines (sedation, dizziness, myorelaxation, interactions with alcohol and particularly the risk of dependence with chronic use) have led to an increased effort to develop novel non-benzodiazepine sedatives devoid of the unwanted side-effects associated with the classical benzodiazepines (Ebert et al., 2006).

GABA\(_A\) receptors containing \(\alpha_4\) or \(\alpha_6\) subunits have a different pharmacological profile characterized by a low affinity of the prototypical 1,4-benzodiazepines (e.g. diazepam and flunitrazepam) and have therefore been termed “diazepam-insensitive” GABA\(_A\) receptors (reviewed by Möhler et al., 2002). These diazepam-insensitive \(\alpha_4\) or \(\alpha_6\) containing GABA\(_A\) receptors have a high sequence similarity to diazepam-sensitive \(\alpha_1\), \(\alpha_2\), \(\alpha_3\) or \(\alpha_5\) containing receptors, but it was revealed that one amino acid in the N-terminal domain was responsible for benzodiazepine sensitivity. Histidine 101 in \(\alpha_1\), \(\alpha_2\), \(\alpha_3\) or \(\alpha_5\) is a arginine in \(\alpha_4\) and \(\alpha_6\) and mutating this arginine to a histidine in \(\alpha_4\) and \(\alpha_6\) made these receptors sensitive to benzodiazepines when expressed with \(\beta\) and the \(\gamma_2\) subunit in various expressions systems in-vitro. This His-Arg substitution has no observed effects on the receptor function other than specifically decreasing the affinity for benzodiazepines thousand fold, the sensitivity to GABA and other modulators remain unchanged.

These results with diazepam insensitive GABA receptors lead to the seminal work by Möhler’s group (Rudolph et al., 1999; Löw et al., 2000). They created transgenic mice with GABA\(_A\) receptors composed of either \(\alpha_1\), \(\alpha_2\), \(\alpha_3\) or \(\alpha_5\) receptor subunits insensitive to diazepam (H101R knock-in mutations), thereby probing the significance of individual subunits for the effects mediated by diazepam. These studies have shown that the sedative effect of diazepam are mediated by GABA\(_A\) receptors
containing the \( \alpha_1 \) subunit, while the anxiolytic and myorelaxation effects are probably mediated by the \( \alpha_2 \) containing subtype and not \( \alpha_1, \alpha_3 \) or \( \alpha_5 \) subunit containing GABA\(_A\) receptors. These studies have elegantly shown that specific GABA\(_A\) receptor subtypes also mediate specific effects of benzodiazepines (reviewed by Rudolph and Möhler 2006). Using the same knock-in mice very recent work (Knabl et al., 2008) has shown that GABA\(_A\) \( \alpha_2 \) and \( \alpha_3 \) containing subunits in the spinal cord play a vital role in pain perception, suggesting that selective anxiolytic benzodiazepines (not showing agonistic activity on \( \alpha_1 \) containing receptors) could also be effective as analgesics.

The multiplicities of subtypes raises the possibility of targeting drugs to specific GABA\(_A\) receptor subunit combinations and thereby achieve an improved clinical profile in the treatment of anxiety, epilepsy, insomnia and potentially, pain.

1.2.3 The Na\(_V\)-1.7 ion channel

Voltage gated sodium channels (Na\(_V\)) play a critical role in the initiation and propagation of action potentials in excitable cells. Sodium channels activate in response to membrane depolarization and are responsible for rapid influx of sodium ions during the rising phase of the action potential. Sodium channels are widely expressed in neuronal, neuroendocrine, skeletal muscle and cardiac cells. Ten genes encode Na\(_V\) \( \alpha \) subunits (Na\(_V\) x and Na\(_V\) 1.1 – Na\(_V\) 1.9 see figure 6) and four auxiliary \( \beta \) subunits (\( \beta_1-4 \) single membrane spanning region) have been identified (Catterall et al., 2005).

The Na\(_V\) channels are formed by one \( \alpha \) subunit making the pore of the channel and one or more \( \beta \) subunits. The \( \alpha \) subunit consist of four homologous domains (I-IV) containing the 6 TM domains (S1-S6). The \( \alpha \) subunit is sufficient to form a functional sodium channel, with the voltage sensor in the S4 and with the ion-selectivity filter made by the pore-forming loop between S5 and S6 and a segment for fast inactivation (third intracellular loop between III-S6 and IV-S1, the I (Isoleucine) F (Phenylalanine) M (Methionine) tripeptide motif, see figure 6).

The \( \beta \) subunit is not part of the pore but serves important functions such as modulating the biophysical properties and anchoring of the channel to the plasma membrane (Catterall et al., 2005). The \( \alpha \) subunit also contains all the sites known today for pharmacological modulation including the tetrodotoxin (TTX) binding site (P loop in domain I) and the local anaesthetic binding site (intracellular S6 in domain IV). Sodium channels can be separated pharmacologically by their sensitivity to TTX, and most Na\(_V\) are blocked by nanomolar concentrations of TTX and termed TTX sensitive while the Na\(_V\) resistant to micro-molar concentrations of TTX (Na\(_V\)-1.5, Na\(_V\)-1.8 and Na\(_V\)-1.9) are classified as TTX resistant.

A number of sodium channels have been shown to be involved in pain responses including Na\(_V\)-1.3, 1.8 and 1.9 (Lai et al., 2004; Priest and Kaczorowski 2007) but within the latest year Na\(_V\)-1.7 have received enormous attention in the pain field (reviewed by Dib-Hajj et al., 2007). The role of the sodium channel subtype Na\(_V\)-1.7 (SCN9A) in pain has been demonstrated very convincingly in humans due to the
recent discovery of loss of function mutations in the SCN9A gene in families with complete inability to sense pain (Cox et al., 2006), and gain of function mutations in the SCN9A gene leading to the painful inherited human neuropathy known as erythromelalgia (reviewed by Drenth and Waxman, 2007).

This initial study by Cox and co-workers identified a Pakistani family with members who cannot feel pain, but otherwise appeared normal. Such families were also reported from Canada (Ahmad et al., 2007) and other parts of the world (Goldberg et al., 2007) and the pain insensitivity has been shown to be present at birth and termed congenital indifference to pain (CIP). The genetic analysis has shown the trait is linked to a region of chromosome 2 that contains the SCN9A gene. Sequence analysis of SCN9A showed several distinct homozygous nonsense mutations in the affected individuals in the different families. All of these mutations lead to truncated non-functional Na\textsubscript{V}-1.7 channels and these CIP patients can be considered as human Na\textsubscript{V}-1.7 knock-outs.

The distribution of Na\textsubscript{V}-1.7 also supports the important role in nociceptive signalling due to the expression restricted to DRG and sympathetic ganglion neurons (Toledo-Aral et al., 1997). Furthermore, some of the biophysical parameters of Na\textsubscript{V}-1.7
explain why this channel seems to be so vital for the ability to experience pain. In contrast to other TTX sensitive sodium channels, NaV-1.7 have unique inactivation properties, permitting the channel to respond to small depolarisations. These properties have led to the suggestion that NaV-1.7 functions as a “threshold” channel amplifying generator potentials and thereby setting the gain in nociceptors when expressed with other sodium channels (for review see Dib-Hajj et al., 2007).

These findings in addition to animal knockout models have pointed to NaV-1.7 as a primary drug target and a specific NaV-1.7 antagonist is expected to be a powerful analgesic. Currently no selective NaV-1.7 inhibitors are known but several major pharmaceutical companies are now attempting to develop such molecules. Sodium channels have been known as a drug target for many years (Lidocaine was developed 1947), but all known sodium channel inhibitors suffer from lack of specificity, and also act on the sodium channels expressed in the heart (NaV-1.5) and brain (NaV-1.2), thus suffering from serious side effects. The next few years will show if it is possible to develop sodium channel inhibitors that are specific for sodium channels specifically involved in pain like NaV-1.7, NaV-1.8 and NaV-1.3.

1.2.4 The TRPM8 ion channel

The TRP (Transient Receptor Potential) superfamily of channels shares the common features of six transmembrane domains and permeability to cations. However TRP channels are exceptional among the known families of ion channels in that they display a large diversity of cation selectivities and activation mechanisms. TRP channels are expressed and function in a great variety of multicellular organisms, including fruit flies (Drosophila), worms, zebrafish, mice, and humans. The TRP superfamily is divided into groups 1 and 2, which are further divided into seven subfamilies (Figure 7). The group 1 TRPs consist of five subfamilies, which bear the strongest sequence homology to the founding member of the superfamily, Drosophila TRP (for review see Clapham 2003; Venkatachalam and Montell 2007).

Starting with the discovery of the vanilloid receptor type-1 (TRPV1) in 1997 (Caterina et al., 1997), the founding member of the mammalian family of TRP channels, a completely new family of ion channels emerged. TRPV1 was shown to be a calcium permeable channel activated by high temperatures (>43°C), low pH and the “hot” ingredient in chili pepper, capsaicin (for review see Julius and Basbaum, 2001). Additional heat sensitive TRP channels were discovered the following years, and the term thermosensitive TRP channels were introduced (Patapoutian, et al., 2003).

Whereas heat sensitive TRPs have been known for some years, the proteins responsible for cold sensitivity were unknown until 2002, when the first example of a protein directly activated by cool temperatures was demonstrated with the cloning and characterisation of the TRPM8 channel (Peier et al., 2002; McKemy et al., 2002). Furthermore the TRPM8 channel expression in a subpopulation of neurones in DRG and trigeminal ganglia correlates well with responses to cooling and menthol (Nealen et al., 2003; Thut et al., 2003; Babes et al., 2004). With these findings TRPM8 was identified as a candidate for a molecular mediator of cold perception and possibly cold
mediated pain, but in-vivo confirmation of this hypothesis had been lacking until 2007 with the creation of TRPM8 knockout mice (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007).

Figure 7. The TRP superfamily. (a) Single members from each of the five group 1 subfamilies. (b) Single members from each of the two group 2 subfamilies. The following domains are indicated: A, ankyrin repeats; cc, coiled-coil domain; protein kinase domain; TRP domain. Also shown are transmembrane segments (vertical rectangles) and pore loop (P), allowing the passage of cations (+++). (c) Composition of the TRP superfamily in worms, flies, mice, and humans. Human TRPC2 is a pseudogene and is not counted and TRPP1-like proteins are not counted.

These knockouts all demonstrated the involvement of TRPM8 in many aspects of cold signalling including cool and noxious cold perception, injury-evoked cold sensitisation and cooling-induced analgesia, suggesting that TRPM8 antagonists could potentially be developed as new analgesics. Interestingly, TRPM8 agonists have been shown to suppress mechanical and heat nociception in the CCI animal model of neuropathic pain (Proudfoot et al., 2006) indicating that a TRPM8 agonist would be
analgesic depending on the pain condition. Patients with neuropathic pain often suffer from cold allodynia, implicating a role for TRPM8 in human pain conditions.

TRPM8 was independently cloned from rat (McKemy et al., 2002) and mouse (Peier et al., 2002a), in addition to low temperature the cooling compounds menthol, icilin and eucalyptol were shown to activate the TRPM8 channel. TRPM8 belongs to the ‘long’, or melastatin, subfamily of the TRP channel family of ion channels, and shows pronounced outward rectification with a relatively high permeability for Ca\(^{2+}\) ions, and little selectivity between monovalent cations.

The average thresholds for TRPM8-expressing cells were first reported to be 21.8° (Peier et al., 2002) and 27.1° (McKemy et al., 2002), with some thresholds as low as 10° and none above 30° (McKemy et al., 2002; Peier et al., 2002). Subsequently, two types of cold-sensitive rat trigeminal ganglion neurons that express TRPM8 were reported, the more sensitive of which had an average threshold of 30.1°C (Thut et al., 2003). Treatment with menthol, or with cold temperatures, is a traditional method of pain relief (Green and Mcaniffe, 2000; Galeotti, et al., 2002), but little is known about the underlying analgesic mechanisms, thus the TRPM8 channel represents the first specific molecular target for menthol.

Menthol was found to modulate the response of TRPM8 to cold and to shift its threshold to higher temperatures (McKemy et al., 2002), and high temperatures (30°C) could antagonise the menthol response. Accordingly, menthol can be considered as an allosteric modulator of temperature activation (or the other way around) in classical pharmacological terms. The other cooling agent, icilin, initially reported to potently (EC\(_{50}\) ~360nM) activate TRPM8 (McKemy et al., 2002), has later been shown to posses a more complex mode of activation, which is calcium dependent (Chuang et al., 2004). Another paper (Andersson, et al., 2004) has shown that when TRPM8 is activated by cold or icilin, acidification by [H\(^+\)] inhibits the response, while activation by menthol is unaffected. In addition (Bandell, et al., 2004) have shown that the menthol and cold activation of TRPM8 can be blocked by the specific phospholipase C (PLC) inhibitor U73122, leading the authors to suggest that a basal PLC activity is required for full activation of the TRPM8 channel, illustrating a complex and far from fully understood activation mechanism for this channel.

To further complicate the activation mechanism(s) for the TRPM8 channel, it has been shown to be voltage sensitive. TRP channels are is generally considered voltage independent channels, i.e. they do not change their activation dependent on the membrane potential, but the TRPM8 channels show some degree of voltage dependence (Voets, et al., 2004; McKemy et al., 2002; Peier et al., 2002). Currents recorded from HEK293 cells expressing human TRPM8 when clamped at fixed membrane potentials, shows that a membrane potential of ~100mV is needed to activate the channel at 37°C (Voets, et al., 2004). But lowering the temperature to 15°C activates the channel already at a membrane potential of ~50mV, and a similar effect is seen using increasing concentrations of menthol instead of lowering the temperature.

These data suggest that the TRPM8 channel is an ion channel with a very complex set of activation mechanism(s), apparently allosterically coupled to each
other, cold activation – ligand activation – voltage activation. The voltage activation would most likely not play a role under normal physiological conditions, but if a cold stimuli or endogenous menthol-like compound, or a combination of those two would act upon the channel, the channel could be activated like the classical voltage-gated ion channels.

At the moment there are no specific TRPM8 antagonists known and therefore the development of specific antagonists in order to pharmacologically validate the role of TRPM8 in pain would be very valuable.

1.3 SCREENING ASSAYS FOR ION CHANNELS

Recent advances in laboratory automation and ion channel screening technology have significantly increased the throughput of ion channel assays (Mattheakis and Savchenko 2001; González and Maher 2002; Bennett and Guthrie 2003; Molokanova and Savchenko 2008). These technological advances have affected both HTS and post-HTS activities in ion channel drug discovery. Whereas ion channel assays only a few years ago were mainly used to characterize a few, highly advanced leads in later stages of the drug discovery process, a number of ion channels technologies are now available to identify new chemical starting points for medicinal chemistry in the lead generation process. However, careful assay configuration to model drug-target interactions in a biologically relevant manner remains an essential consideration.

Apart from increasing throughput, the recent developments in post-HTS screening technology have also enabled researchers to assess ion channel function and pharmacology with highly controlled, physiologically relevant stimulus paradigms that test for example for state-dependent interaction of compounds. This will very likely lead to a paradigm shift within the pharmaceutical industry since it opens up the study of ion channels in today’s rationale drug discovery process.

The attractiveness of a specific screening technology for drug discovery purposes is determined by its amenability to miniaturization, reliability, reproducibility and low costs. When it comes to ion channel screening platforms the detection of fast activation and inactivation, identification of use- and state-dependence, and good correlation with conventional electrophysiology are additional criteria. Despite recent developments, a high throughput ion channel-screening platform applicable to all channels meeting the above characteristics is not available. Voltage-gated ion channels present a particular challenge for HTS, since - as the name implies - the activator is a change in membrane potential. Thus, an ideal screening technology would be a system that allows voltage control in a high throughput manner. However, presently there is no such instrument commercially available.

Overall, present screening approaches can be divided into four types of assays; ligand binding, ion flux, optical or fluorescence, and automated electrophysiology. Only the three latter approaches are functional assays, which means that the results
obtained in these assays report, directly or indirectly, on the functionality of the investigated channel as an ion-conducting pore.

1.3.1 Binding assays

During the 1970s radio-ligand binding assays were used to study the ability of novel drugs to displace the radio labelled ligand, in order to determine if the drug can bind to the ion channel of interest. This methodology, together with computer based pharmacophore modeling during the 1980s lead to quantitative structure activity relationship (SAR) studies for drugs interacting with ion channels. In combination with site directed mutagenesis studies on cloned ion channels, these techniques have improved the understanding of ion channel molecular pharmacology tremendously.

Binding assays generally measure the proportion of a ligand (radioactive or fluorescently labelled) bound to the receptor at equilibrium. To achieve this the receptor bound ligand have to be separated from the un-bound ligand (and non-specific bound ligand). Before the introduction of cloning and expression of single receptor subunits in the late 1980s, binding assays were always done on native tissue expressing the receptors investigated. But now most binding assays use membrane preparations of cells over expressing recombinant specific receptor subtypes. Although these methods give detailed information about the binding mode of the drugs in question, the pharmacological properties are very difficult to predict i.e. agonists (full, partial or inverse) or antagonists based on binding studies.

1.3.2 Flux assays

Atomic absorption spectroscopy (AAS) is a technique used to determine the concentration of a particular metal in a sample, where the concentration of over 50 different metals in solution can be analyzed. AAS uses the absorption of light to measure the concentration of gas-phase atoms. The analyte atoms or ions must be vaporized in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels and the analyte concentration is determined from the amount of absorption.

Recent advances in AAS technology allowing detection of small volumes of biological samples have made it possible to develop these assays for HTS purposes of ion channel targets (Stankovich et al., 2004). The Ion Channel Reader series from Aurora Biomed is currently the most used AAS platform for ion channel screening. Traditionally ion flux assays have utilized radioisotopes such as \(^{86}\)Rb for potassium channels. AAS now allow the use of non-radioactive rubidium (Rb\(^+\)) or lithium (Li\(^+\)) as surrogate ions in potassium and sodium channel assays, respectively. The use of conventional AAS to detect Rb\(^+\) in samples from Rb\(^+\) efflux assays has been described for a medium throughput screening of compounds acting on potassium channels (Scott et al., 2003). Similarly, Li\(^+\) can be used as a surrogate ion for sodium to measure activity of sodium-permeable ion channels such as voltage-gated sodium channels (paper III in this thesis).
1.3.3 Optical assays

Starting in 1994 with the development of the FLIPR (Fluorometric Imaging Plate Reader) the development in fluorescent-based techniques and microplates for cell based assays has been enormous (Schroeder and Neagle 1996). With the FLIPR high throughput screening became possible on living cells allowing studies on intracellular calcium concentrations \([\text{Ca}^{2+}]_i\) and membrane potential (Sullivan et al., 1999; Chambers et al., 2003). The FLIPR-based assays can be used to determine the pharmacological activity of novel drugs acting on various types of ion channels, giving reasonably good correlation with results obtained from patch-clamp electrophysiology.

Fluorescent dye assays are commonly based on two principles. Firstly, the fluorescence properties of the reporter dye can be affected by interacting with a specific ion of interest. The most common dyes are sensitive to calcium, but dyes sensitive to chloride, sodium, or hydrogen ions are also available. Using such ion-sensitive dyes it is possible to run an HTS on the majority of ligand-gated channels, but also voltage-gated calcium channels. Typically the assay will be optimized to detect influx of the ion directly through the channel of interest. However, the approach can also be used more indirectly, for example to follow changes in intracellular pH caused by ion channel activity.

A second principle is based on using dyes that alter their fluorescence properties upon changes in membrane potential. Such fluorescent dyes that are sensitive to changes in membrane potential have been known for some time but have been optimized significantly during the last years. Due to these improvements, it is now possible to measure changes in membrane potential in HTS format (384 and 1536 well plate format) using fluorescent plate readers with integrated liquid handling like the FLIPR and FLIPR-tetra from Molecular Devices or the FDSS6000 from Hamamatsu.

The FLIPR system uses a 488 nM argon laser for excitation of the fluorescent dye. Emission intensity at 550 nM increases when the membrane depolarizes. The Hamamatsu and FLIPR-tetra instrument can run dual wavelength excitation, allowing measurement of fluorescence resonant energy transfer (FRET). The obtained ratiometric signal increases in response to membrane depolarization. FRET is obtained between a membrane-bound donor molecule and a mobile voltage-sensitive acceptor (for review see Molokanova and Savchenko 2008). The donor is a coumarin-linked phospholipid that binds to the outer leaflet of the plasma membrane, whereas the acceptor is a negatively charged oxonol that partitions across the plasma membrane as a function of membrane potential. Ratio-metric approaches have the clear advantage of compensating for several drawbacks like variations in dye loading and cell density.

Aurora Biosciences Corporation has developed a voltage-sensitive probe/ion channel technology (VIPR) based on FRET as described above. VIPR is an integrated liquid handler and kinetic fluorescence reader for 96 or 384 well microtitre plates that combines an eight-channel liquid handler, a microplate-positioning stage and a fiber-optic illumination and detection system (González and Maher 2002). A further development of the VIPR to include electrical stimulation is the E-VIPR, developed by...
Vertex Pharmaceuticals (Huang et al., 2006) allowing some of the stimulation control and temporal resolution offered by electrophysiology. The platform provides an extracellular electrode array in standard 96 or 384 well microtiter plates and enables the determination of use-dependence of compounds.

1.3.4 Automated electrophysiological assays

The direct measurement of ion channel activity became possible in the early 1980s with the development of patch-clamp electrophysiology (Sakmann and Neher 1995). With the patch-clamp technique, the activity of a single ion channel or the ion channel activity of a whole cell can be investigated giving very detailed information regarding ion channel function and pharmacology at single cell level. Today the patch-clamp technique is still the “golden-standard” to study ion channel pharmacology, but this method has the major draw back of being extremely time consuming and requiring highly trained operators. Within the last 4 years a remarkable development in automated electrophysiology has occurred, thereby opening new horizons in ion channel discovery.

With the introduction of the Ionworks-HT™ instrument in 2003 it became possible to obtain electrophysiological data on hundreds of compounds/day (Schroeder et al., 2003). The Ionworks-HT instrument is an integrated platform consisting of computer-controlled fluid handling, recording electronics, and processing tools capable of voltage clamp whole-cell recordings from up to 384 individual cells per experiment (Schroeder et al., 2003). To establish a recording, the system uses a planar, multiwell substrate: the PatchPlate. The robotic liquid system positions cells into a well with a hole separating the two fluid compartments in each well of the substrate. Voltage control and current recordings from the cell membrane are made subsequent to gaining access to the cell interior by applying a permeabilizing agent to the intracellular side. Based on the multiwell design of the PatchPlate, voltage clamp recordings of up to 384 individual cells can be made in less than 40 minutes and are comparable to measurements made using traditional electrophysiology techniques. Because the system is based on single cell measurements, the system have been set up to test each compound on four individual wells. This ensures that results are obtained for every compound tested, but effectively make the IonWorks-HT operate in a 96 well format, as only a maximum of 96 individual compounds can be tested in one experiment.

A second generation of the IonWorks-HT, the IonWorks-Quattro, has recently entered the market. It pioneers the population patch-clamp principle, in which the recordings from several cells are averaged. In the case of IonWorks-Quattro, 64 apertures are present in each well of the recording plate (PPC-plate). The recorded voltage-clamped current in this system thus represents the average of the current generated by up to 64 individual cells. When compared to IonWorks-HT, this second-generation platform approach increases throughput by a factor of 4, in addition to improving data-consistency in screening voltage-gated ion channels (Dale et al., 2007). With IonWorks-Quattro it is now possible to screen voltage-gated ion channels in a true 384 well format, allowing more than thousand compounds per day to be tested. Considering the fact that only five years ago electrophysiological data from voltage-
gated ion channels had to be generated through manual patch-clamp one cell at a time, this truly is a revolution in the field.

PatchXpress was the first platform introduced to address both voltage-gated and ligand-gated ion channels. The platform has 16 parallel amplifiers using a 16 well substrate (SealChip16) with one small aperture per well. It is an open-well format that provides access to automation and parallel processing. The PatchXpress system automatically places cells in each well of the 16 well substrate using a single pipette arm. This pipette arm is also used for compound addition later in the experiment. Gigaseals and whole cell recordings are obtained with independent pressure control and data acquisition for each channel. Under optimal conditions, parallel patch clamping of sixteen cells per chip can generate of up to ~100 concentration response curves in one day (Tao et al., 2004).

The QPatch HT is a 48-channel gigaseal patch-clamp system that can be applied to both voltage-gated and ligand-gated ion channels. The system includes 48 individual patch clamp amplifiers and pressure controllers. The platform has eight pipettes attached to the fluid handling robot allowing to cope with the liquid handling demand of the up to 48 simultaneous patch clamp experiments. The individual measurement sites include glass-coated microfluidic channels for minimal compound absorption allowing for accurate pharmacological data. The microfluidic channels require approximately 5 μl of solution with a liquid exchange time of about 100 ms. The platform also utilises an automated cell preparation station where cells are kept in suspension, transferred to the on-board centrifuge, spun down, and resuspended in the extracellular solution to a suitable concentration for optimal success rates (Mathes 2006).
2 AIMS

Ion channels well-known drug targets. However, the techniques used to study ion channel pharmacology have not been particularly applicable to modern drug discovery. The aim of this thesis is to examine the usefulness of both established and novel methods to the discovery and pharmacological characterisation of drugs interacting with ion channels.

Specific aims for each project were to answer the questions:

1 Can flavonoids be fitted into a comprehensive pharmacophore model for the benzodiazepine-binding site of the GABA_A receptor?

2 Do arginine residues in the extracellular N-terminal of the GABA_A receptor participate in the GABA binding pocket?

3 Which HTS assay is best suited to detect Na_V-1.7 modulators in the beginning of a drug discovery campaign?

4 Can the FLIPR technology be used as a HTS assay to find TRPM8 ligands?
3 MATERIALS AND METHODS

Experimental details are given in the individual papers.

3.1.1 Paper I


Protein concentration was determined using the DC protein assay from Bio-Rad Laboratories.

Synthesis of flavone derivatives.

Computational Methods. Geometry optimizations and conformational analyses of the flavones were performed by using the molecular mechanics program MM3(92) developed by Allinger and co-workers.

3.1.2 Paper II

CHO cells (CHO-K1) were transfected with human GABA$\_A$ receptor subunits.

Mutations were introduced to the human $\alpha5$ GABA$\_A$ receptor subunit sequence according to the QuickChange site-directed mutagenesis kit protocol from Stratagene.

Whole-cell patch-clamp recordings on CHO cells expressing the GABA$\_A$ receptor.

3.1.3 Paper III

HEK293 cell line stably expressing human Na$\_V$-1.7.

Perforated whole cell patch clamp recordings on the automated electrophysiological platform IonWorks HT.

Whole-cell patch-clamp recordings on HEK293 cell line stably expressing Na$\_V$-1.7.

Li flux AAS assay.

FLIPR membrane potential assay.

FRET membrane potential assay.
3.1.4 Paper IV

HEK293 cells stably expressing human TRPM8.

Whole-cell patch-clamp recordings on HEK293 cell line stably expressing TRPM8.

FLIPR calcium sensitive fluorescence (Fluo-4) assay.

Two-electrode voltage clamp electrophysiological recordings on *Xenopus* oocytes expressing human TRPM8.
4 RESULTS AND DISCUSSION

4.1 Ligand interactions at the GABA\textsubscript{\text{A}} receptor: pharmacophore modelling and site directed mutagenesis (paper I and II).

Drugs interacting with the GABA\textsubscript{\text{A}} receptor can be used in the treatment of anxiety, epilepsy and insomnia. However a potential liability for these drugs is the addictive, sedative and abuse potential they have. It have been suggested that compounds with partial agonistic efficacy and/or specificity for only some subunits could overcome these side effects (for review see, Rudolph and Möhler 2006). In order to obtain such drugs knowledge of the structural features of the binding sites in the receptor is very helpful. Due to the difficulty of purifying membrane proteins no crystal structure of GABA\textsubscript{\text{A}} receptors are available and structure-based drug design is not directly applicable. We therefore applied pharmacophore modelling and site-directed mutagenesis to study the ligand interactions at a structural level.

In previous studies we used Chinese medicinal herbs to extract and identify several flavonoids as GABA\textsubscript{\text{A}} receptor ligands (Ai et al., 1997). In paper-I we extended these findings by synthesising flavonoids to investigate their structure activity relationships using a \textsuperscript{3}H-flumazenil binding assay to rat cortical membranes in-vitro.

A comprehensive pharmacophore model for the BzR based on structure-activity relationship studies for 136 different ligands from ten structurally different classes of compounds has been developed (Zhang et al., 1995). This model assumes that BzR agonists, antagonists and inverse agonists share the same binding pocket. The pharmacophore model has successfully been employed in the design of novel BzR ligands (Diaz-Arauzo et al., 1991) and used in the structure-activity analyses of novel ß-carboline ligands (Da Settimo et al., 1998). Although the pharmacophore model is based on ligands from a large number of different classes of compounds, flavonoids were not included in the development of the model.

In paper-I we show that flavonoids with high affinity for the benzodiazepine receptor spanning the whole efficacy range from agonists (\textit{1q}) to inverse agonists (\textit{1l}) can be synthesised. The receptor binding properties of the flavonoids studied can successfully be rationalised in terms of a comprehensive pharmacophore model. The proposed binding mode of compound (\textit{1q}) is shown in Figure 8. H1 and A2 are hydrogen bond donor and acceptor sites, respectively, whereas H2/A3 is a bifunctional hydrogen bond donor/acceptor site. L1-L3 are three lipophilic pockets and S1-S3 denote regions of steric repulsive ligand-receptor interactions (receptor-essential volumes).

The main conclusions from this structure activity analysis of flavonoids are that in order to bind to the benzodiazepine site, the flavone skeleton should be planar or close to planar. Small substituents such as methyl and bromine in the 6-position significantly increase the affinity, whereas a 4'-NO\textsubscript{2} group significantly decreases the
affinity. A 3’-NO$_2$ or 3’-methyl group (directed “downwards” in Figure 8) strongly increases the affinity making this substituent position of great interest for further investigations. None of the studied flavonoids was able to interact with the hydrogen bond accepting site A2, although this has been found to be an important interaction site for compounds that display potent inverse agonism. Despite this, 6-methyl-3’-nitroflavone was shown to be a high affinity ligand with a $K_i$ of 5.6 nM and displaying inverse agonism, predicted from the GABA ratio.

![Figure 8](image)

**Figure 8.** Proposed binding mode of 6-methyl-3’,5-dinitroflavone (1q) in the pharmacophore model.

On the basis of the results of these studies the model was further developed employing a large number of flavone derivatives not included in this work and of the refined pharmacophore model, 5’-bromo-2’-hydroxy-6-methylflavone was designed (Kahnberg et al., 2002), showing the highest binding affinity ($K_i$ 0.9 nM) to the BzR in-vitro reported for a flavone derivative. Furthermore the pharmacophore model has been converted into a Catalyst pharmacophore model (Kahnberg et al., 2004). This Catalyst pharmacophore model was used as a search query for 3D database searching in an attempt to find new classes of compounds with affinity for the benzodiazepine site of the GABA$_A$ receptor. From these searches, several novel compounds were found with the most potent compound showing a $K_i$ value of 121 nM.

Following the work described here several research groups have been able to generate synthetic flavone derivatives with higher affinities for the GABA$_A$ receptor, by means of the synthesis of small organic molecules libraries prepared by combinatorial chemistry, performed on solid or solution phases, and assisted with the molecular modeling of the flavonoid binding to the BzR binding site (Huang et al., 2001; Marder and Paladini, 2002; Kahnberg et al., 2002; Hong and Hopfinger, 2003; Kahnberg et al., 2004). Furthermore the original model has been extended and now includes the flavonoids (Clayton et al., 2007).

Ligand binding properties of the GABA$_A$ receptor have been investigated by site-directed mutagenesis of individual subunits using binding studies and electrophysiological measurements as assays for analysing the effects of point mutations on the receptor function (reviewed by Davies et al., 1996; Sigel et al., 2006).
A structural analysis of several GABA<sub>A</sub> receptor agonists and antagonists has suggested a GABA binding site model (Galvez-Ruano et al., 1995). The model predicts that positively charged arginine residues participate in hydrogen bonding to the carboxylate group of the GABA molecule.

In paper-II we tested this hypothesis by mutating arginine residues potentially contributing to the formation of a GABA binding pocket. Thus, six arginines conserved in all human GABA<sub>A</sub> receptor α subunits (arginine 34, 70, 77, 123, 135 and 224, numbering without signal sequence) as well as two non-conserved arginines (79 and 190) in the extracellular, N-terminal segment of the α5 subunit were substituted by lysines. The individual α5 subunit mutants were co-expressed with human β2 and γ2s GABA<sub>A</sub> receptor subunits in CHO cells by transient transfection.

Electrophysiological whole-cell patch-clamp recordings showed that, of the eight arginine residues tested, only the two arginines at position 70 and 123 appear to be essential for GABA-gated chloride current, since the EC<sub>50</sub> values of the two mutant constructs increase more than 100-fold compared to wild-type α5β2γ2s GABA<sub>A</sub> receptors. However, both diazepam and allopregnanolone modulation and pentobarbital stimulation properties are unaffected by the introduction of lysines at position 70 and 123.

In support of our data, earlier reports have shown that each of the arginines (70 and 123) is located in structural regions in which individual amino acids cannot be changed without severely affecting the GABA<sub>A</sub> receptor function. The phenylalanine at position 64 in the α1 subunit and the corresponding phenylalanine at position 68 in the α5 subunit have been exchanged with leucine, resulting in strong rightward shifts of the GABA EC<sub>50</sub> curves (Sigel et al., 1992; Buhr et al., 1996). It is interesting that the arginine residue (R70 in α5, R68 in α1) is conserved among the α and β subunits of the GABA<sub>A</sub> and GABA<sub>C</sub> receptors, respectively, and that arginines are found at the complementary sites of the glycine receptor α subunits.

Affinity-labeling and mutation studies of nicotinic acetylcholine receptor subunits have identified four discontinuous loops (A–D, of which loop D contains R70), which are important for bridging together the ligand binding site. The existence of structural and sequence homologies of these four loop regions among subunits from the various receptor types of the ligand-gated ion channel receptor superfamily has suggested that the four loops and the folding of these into the ligand binding site are common mechanisms shared by all members of the superfamily (for review, see Campagna-Slater and Weaver 2007). Mutation experiments have also shown that a tryptophan at position 64 in the α7 nicotinic acetylcholine receptor subunit (complementary to phenylalanine 68 in the GABA<sub>A</sub> α5 receptor subunit) is directly involved in ligand binding (Corringer et al., 1995), all pointing to the crucial function of the region in ligand binding.

Our results could be explained structurally by a study published at the same time our results came out (Boileau et al., 1999). It suggests that the region Y59–S68 within the GABA<sub>A</sub> α1 subunit (equivalent to Y63–S72 in the α5 subunit) forms a β-strand in which the residues F64, R66 (corresponding to R70 in the α5 subunit), and S68 are
facing into the GABA binding site, where the residues probably interact directly with the GABA molecule (Boileau et al., 1999). Based on these results the authors suggested a binding model for GABA (Figure 9).

**Figure 9.** Theoretical structure of the agonist-binding site of the GABA$_\alpha$ receptor.

A) Molecular model of the GABA binding site pocket, with residues from the $\alpha 1$ subunit (Y59-K70, left) and the $\beta 2$ subunit (Y157-T160 and T202-Y205, right) surrounding a GABA molecule. $\alpha 1$ residues are arranged in an idealized $\beta$-strand conformation, with MTSEA-Biotin reactive side chains highlighted in white (reactive but not protected by agonist) or magenta (protected by agonist). $\beta 2$ subunit segments are shown in $\alpha$-helical conformation. Selected oxygens (red) and nitrogens (cyan) are depicted for orientation and to show charged moieties.

B) Model of a covalently modified $\alpha 1$S68C mutant receptor binding site. After reaction with MTSEA-Biotin, the introduced cysteine forms a covalent disulfide bond with $-\text{SCH}_2\text{CH}_2\text{-Biotin (-S-Biotin)}$. Orientation of the $-\text{SCH}_2\text{CH}_2\text{-Biotin}$ is purely speculative and is shown in an extended conformation. Sulfur atoms are shown in orange. Peptide chains were created using Sybyl software (Tripos Associates) and rendered using WebLab (Molecular Simulations) and Adobe Photoshop (Adobe Systems) software. Cartoon depictions of other molecules were created using ISIS (MDL Information Systems) chemical modeling software (from Boileau et al., 1999).

The other critical arginine residue at position 123 in the $\alpha 5$ subunit is not located in any of the proposed four loop regions. However, this residue is conserved among all the GABA$_A$ receptor $\alpha$ and $\rho$ subunits, and glycine receptor $\alpha$ subunits. An earlier study has shown that lysine substitution of the homologous arginine in the GABA$_A$ $\alpha 1$ subunit (R120) results in a 180-fold higher GABA EC$_{50}$ value of $\alpha 1$-$\beta 2$-$\gamma 2s$ GABA$_A$ receptors (Westh-Hansen et al., 1999), suggesting that the arginine residue may play an important role in either forming the GABA binding site or in making a crucial interaction between different loops of the receptor. The importance of residue 123 is also indicated by the observation that the complementary amino acids in the acetylcholine receptor $\gamma$ and $\delta$ subunits are involved in subunit-specific ligand binding selectivity (Sine et al., 1995).
4.2 Development of functional HTS assays for \( \text{Na}_V \) and TRPM8 ligands (Paper III and IV).

As stated in the introduction, ion channels are challenging targets in the early phases of the drug discovery process, especially due to the lack of technologies available to screen large numbers of compounds in functionally relevant assays. The electrophysiological patch-clamp technique, which is the gold standard for studying ion channels, has low throughput and is not amenable to HTS. However, for random HTS of compounds against ion channel targets, a number of functional cellular assays have become available during the last few years (see introduction).

Successful drug discovery relies on reproducibility of assays and a screening cascade that incorporates different assays to increase confidence and knowledge about the mechanism of action of the studied compounds. Consequently, different methods and read-outs of primary and secondary screening assays should identify the same compounds as active on a given target. Although each of the different ion channel screening technologies described above allows identification of compounds active on an ion channel of interest, it is not given that they will identify the same compounds. The read-outs are different between assays and can measure channel activity indirectly, for example when detecting changes in membrane potential by fluorescent dyes. Thus, it is a common practice to compare results obtained with different assays on the same target prior to embarking on a major drug discovery effort.

In paper-III we use the sodium channel \( \text{Na}_V-1.7 \) stably expressed in HEK293 cells and compare 3 HTS assays, a \( \text{Li}^+ \) flux atomic absorption spectroscopy (AAS) assay, a FLIPR membrane potential assay and a FRET based membrane potential assay to an automated electrophysiological assay (the Ionworks-HT™ platform) and characterize eleven known sodium channel inhibitors. Activation of voltage-gated sodium channels leads to sodium influx across the cell membrane resulting in changes in membrane potential. In the study we have exploited both the actual ion-flux as well as the change in membrane potential for the development of a functional HTS assay using a \( \text{Li}^+ \)-influx based AAS assay and a membrane potential dye FLIPR assay, respectively.

Furthermore we tested a set of 13000 compounds selected from pharmacophore modelling. The active compounds, defined as inducing more than 40% inhibition of \( \text{Na}_V-1.7 \) signal at 10 \( \mu \text{M} \) compound concentration in either the \( \text{Li}^+ \)-flux assay or FLIPR assay, were then further studied with the IonWorks electrophysiology platform.

Voltage-gated sodium channels can exist in multiple conformational states including resting, open and inactivated (closed and not able to open). At equilibrium, the channels mainly exist in the resting state in hyperpolarized membranes and conversely predominantly in the inactivated state in depolarized membranes. Channel activation occurs immediately after a sufficiently large change in membrane potential in the depolarizing direction take place. This will cause a transition of the channel from the resting state to a short-lived open state, followed by inactivation of the channel. Therefore, in order for an assay to detect channel activity, the resting membrane
potential of the cell must be sufficiently hyperpolarized to maintain channels in the resting state prior to activation by membrane depolarization. This can easily be accomplished in voltage-clamp electrophysiological assays where membrane potential can be precisely controlled.

However, both in the influx and membrane potential dye assays the resting membrane potential of the cell cannot be controlled. Furthermore, most standard cell lines that are being used for expression of ion channels, like HEK and CHO have lower hyperpolarized resting membrane potentials than excitable cells and, as a consequence, a smaller population of channels will exist in the resting state. Therefore, we found it necessary to enhance the measured response in both the Li⁺-flux based AAS assay and the fluorescence–based membrane potential assay by prolonging the channel open state with extracellular scorpion venom (1 μg/ml) and simultaneously preventing channel inactivation using 25 μM veratridine.

Using these pharmacological tools, we were able to screen the set of 13000 compounds with both assays. Various statistical parameters have been applied to evaluate assay sensitivity and specificity. A widely used statistical parameter is the Z’ factor, which provides a measure of the reliability of hit selection based on the dynamic range and intrinsic variability of the assay (Zhang et al., 1999), and this has now become a standardized measurement of the assay quality in the HTS community. The Z’-factor has been defined as the ratio of the separation band to the dynamic range of the assay, based on the mean of the positive control (maximum response, including standard deviation) and the mean of the negative control (buffer response, including standard deviation) data of the assay. The reproducibility of data was much higher with the Li⁺-flux assay (z’=0.62) as compared to the FLIPR membrane potential assay (z’=0.33).

Using the FLIPR assay, 1933 compounds were identified as active, whereas the Li⁺-flux assay identified 1604 compounds as active (Figure 10). 982 of these

![Figure 10](image-url)  
**Figure 10.** Overlap of Na⁺-1.7 channel blockers in IonWorks HT (IW), Li⁺-flux (AAS), and membrane potential (MP) assays.

Using the FLIPR assay, 1933 compounds were identified as active, whereas the Li⁺-flux assay identified 1604 compounds as active (Figure 10). 982 of these
compounds were identified by both assays, and almost half (48\%) of the compounds active in both assays could be confirmed using IonWorks (467 compounds). In contrast, only 6\% (58) of 910 additional FLIPR-active compounds were confirmed using IonWorks, while 24\% (138 of 582) additional actives in the Li\(^{+}\)-flux assay were confirmed in the electrophysiology assay. Due to limited compound availability not all active compounds could be re-screened, explaining the slightly higher numbers of actives identified in the initial FLIPR (1933 actives) and Li\(^{+}\)-flux assays (1604 actives) as compared to the retested compounds in the IonWorks assay (1892 and 1564, respectively).

Our results show that all 3 HTS assays can be used for the identification of Nav-1.7 inhibitors, but as an HTS assay the Li\(^{+}\)-flux assay is more robust than the FLIPR and FRET based membrane potential assays. Furthermore, there was a better correlation between the IonWorks assay and the Li\(^{+}\)-flux assay regarding the IC\(_{50}\) values of the sodium channel inhibitors investigated, compared to the FLIPR and FRET assays. This project describes the first comparison between all the HTS assays available today to study voltage gated sodium channels. Overall the results indicate that the Li\(^{+}\)-flux based AAS assay is superior to the membrane potential fluorescent dye FLIPR assays in predicting activity in the electrophysiology assay. However, both HTS assays identify a significant number of false positives making confirmation of identified compounds using IonWorks paramount.

In paper-IV we used HEK293 cells stably expressing human TRPM8 channels to develop a FLIPR based HTS assay using the calcium sensitive dye Fluo-4 using both menthol, icilin and cold as agonists. Consistent with the reported pharmacology of TRPM8 (McKemy et al., 2002; Peier et al., 2002), menthol, and icilin increased [Ca\(^{2+}\)]\(_i\) in HEK293 cells stably expressing TRPM8. As expected, responses of both agonists were dependent on extracellular calcium and removing all extracellular calcium prevented any agonist response by icilin, whereas menthol produced a slight increase in [Ca\(^{2+}\)]\(_i\). A previous finding showed that icilin activation of the TRPM8 channel is completely dependent of calcium and Ca\(^{2+}\) ions was suggested to be a co-agonist (Chuang et al., 2004), whereas menthol can activate the channel in the absence of calcium ions, this is in agreement with the observations made here.

Increasing the temperature to 37\(^{\circ}\)C in the FLIPR experiments decreases [Ca\(^{2+}\)]\(_i\), suggesting that some TRPM8 channels are active at room temperature, and by increasing the temperature they close. This experiment also illustrates the complex allosteric coupling between temperature activation and agonist activation reported for TRPM8 (McKemy et al., 2002; Peier et al., 2002; Bandell, et al., 2004; Voets, et al., 2004; Chuang et al., 2004). The agonists menthol and icilin seem to lower the temperature activation threshold in an allosteric manner, e.g. the channel activates at higher temperatures with either of the agonists present and as a consequence, the concentration response curves for both agonists were shifted to the right at higher temperatures. Additionally, the agonist icilin seems to have a more complex activation mechanism(s) on the TRPM8 channel, therefore menthol was used as the agonist when screening for novel antagonists.
Capsazepine and BCTC were the only TRPV1 antagonists that showed any ability to antagonise TRPM8 albeit with much lower potency than that reported for the TRPV1 channel (capsazepine IC$_{50}$ 2.6 µM, Smart et al., 2001), (BCTC IC$_{50}$ 35 nM, Valenzano et al., 2003). Capsazepine shifted the menthol concentration response curve to the right without affecting the maximal response suggested that capsazepine is a competitive inhibitor of the menthol response. A previous report using the mouse TRPM8 expressed in HEK cells (Behrendt, et al., 2004), also identify capsazepine (IC$_{50}$ ~18µM) and BCTC (IC$_{50}$ ~0.8µM) as antagonists. These authors also used a FLIPR assay with menthol as the agonist, and the differences in potency of these antagonists could be due to species differences, although the EC$_{50}$ value for menthol (~4µM) and icilin (~0.2µM) are in good agreement with the values observed here.

Screening an AstraZeneca library identified hundreds of hits, defined as compounds inhibiting the menthol induced response >50% at a concentration of 10µM. Of these hits, 15 compounds were selected from different structural classes to be further characterised pharmacologically (Table-1). The compounds fully inhibited the menthol induced calcium response with potencies exceeding the reference compound capsazepine by a factor 5-10. Furthermore these antagonists blocked the icilin-induced response with similar IC$_{50}$ values, suggesting that these antagonists are not discriminating against the agonist used.

Table-1

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC$_{50}$ Cold FLIPR [µM]</th>
<th>IC$_{50}$ Menthol FLIPR [µM]</th>
<th>IC$_{50}$ Icilin FLIPR [µM]</th>
<th>IC$_{50}$ Menthol Oocytes [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% inhibition)</td>
<td>(% inhibition)</td>
<td>(% inhibition)</td>
<td></td>
</tr>
<tr>
<td>Az1</td>
<td>0.6 (61%)</td>
<td>1.5 (94%)</td>
<td>4.1 (100%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Az2</td>
<td>0.9 (71%)</td>
<td>2.2 (100%)</td>
<td>2.9 (100%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Az3</td>
<td>4.0 (36%)</td>
<td>2.7 (75%)</td>
<td>-</td>
<td>1.1</td>
</tr>
<tr>
<td>Az4</td>
<td>0.02 (100%)</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>Az5</td>
<td>1.0 (90%)</td>
<td>2.2 (95%)</td>
<td>3.0 (98%)</td>
<td>0.6</td>
</tr>
<tr>
<td>Az6</td>
<td>0.5 (90%)</td>
<td>1.4 (90%)</td>
<td>3.7 (100%)</td>
<td>2.1</td>
</tr>
<tr>
<td>Az7</td>
<td>5.2 (98%)</td>
<td>3.8 (100%)</td>
<td>12.1 (99%)</td>
<td>1.4</td>
</tr>
<tr>
<td>Az8</td>
<td>0.9 (82%)</td>
<td>1.2 (97%)</td>
<td>1.7 (98%)</td>
<td>2.3</td>
</tr>
<tr>
<td>Az9</td>
<td>0.4 (96%)</td>
<td>1.3 (93%)</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>Az10</td>
<td>2.9 (90%)</td>
<td>2.4 (88%)</td>
<td>6.1 (97%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Az11</td>
<td>0.3 (96%)</td>
<td>1.6 (98%)</td>
<td>1.4 -</td>
<td>0.3</td>
</tr>
<tr>
<td>Az12</td>
<td>0.1 (99%)</td>
<td>0.8 (100%)</td>
<td>1.0 (99%)</td>
<td>0.3</td>
</tr>
<tr>
<td>Az13</td>
<td>0.2 (74%)</td>
<td>1.4 (91%)</td>
<td>2.4 (100%)</td>
<td>1.4</td>
</tr>
<tr>
<td>Az14</td>
<td>0.7 (100%)</td>
<td>3.5 (97%)</td>
<td>3.0 (100%)</td>
<td>1.2</td>
</tr>
<tr>
<td>Az15</td>
<td>0.2 (99%)</td>
<td>0.9 (99%)</td>
<td>1.8 -</td>
<td>0.4</td>
</tr>
<tr>
<td>Capsazepine</td>
<td>21.6 (99%)</td>
<td>9.3 (99%)</td>
<td>-</td>
<td>37</td>
</tr>
</tbody>
</table>

To verify the antagonistic properties further, an electrophysiological assay (two-electrode voltage clamp (TEVC)) was established using *Xenopus* oocytes expressing the TRPM8 channel. In this assay the direct function of channel activity is recorded under controlled membrane holding potentials and all antagonists potently (compared
to the reference capsazepine) inhibited the menthol-induced current. Having established that these compounds inhibit the TRPM8 activation independently of agonists and assays used we wanted to investigate if these compounds could antagonize the response to cold temperature.

All of the identified compounds showed concentration dependent antagonistic effects on the cold induced response in TRPM8 expressing HEK293 cells. 11 out of 16 compounds inhibited the cold response fully, but four antagonists (Az1, Az2, Az8, Az13) that failed to inhibit the cold response completely (61%-82%). All antagonists inhibited the menthol and icilin response fully (by 91%-100%), while some of the other antagonists inhibited the cold, icilin and menthol responses approximately equally well. This indicates that it is not the solubility of the four compounds that prevents them from fully inhibiting the cold response. It also opens the possibility that the antagonists that block all response - independent of agonist - may bind in a way that interacts with a common mechanism for channel activation.

In general, the antagonist potency appears to be higher in the assay using cold as the agonist compared to the menthol and icilin assay. This could be due to the lower efficacy of cold stimulations (by ~30%) compared to menthol stimulation. No antagonist that showed inhibitory effect when tested in the menthol assay failed to block the cold response, suggesting that the mechanism for cold activation is related to the mechanism of agonist activation.

In summary, this study has demonstrated that using FLIPR assays both agonists and antagonists of the TRPM8 channel can be pharmacologically characterized. Furthermore the results demonstrate a good correlation between results obtained using TEVC electrophysiological measurements in oocytes and fluorescent assays using HEK cells expressing TRPM8. These results suggest that TRPM8 is a feasible target for antagonists that could lead to novel analgesics.
5 CONCLUDING REMARKS

Ion channels currently represent ~7% of protein drug targets, in contrast to the G protein coupled receptors which represent ~30% or enzymes representing ~50% (Figure 2). Considering the drugability and success of the existing ion channels drugs, it is interesting that they are not better represented as a drug target class. The explanation for this, is that modern drug discovery has not been particularly applicable to ion channels.

Consider that just four years ago the only possibilities for the primary screening of >1000 compounds on ion channels were ligand binding assays or fluorescent dye assays. For the secondary assay the only alternative was manual electrophysiological patch-clamp with a throughput of a few compounds a day. This resulted in a huge gap between the primary and secondary screening assays and as a consequence it was very difficult to support medicinal chemistry in developing new chemical leads. With the latest developments in automated electrophysiological instruments it is now possible to discover new chemical starting points for medicinal chemistry in the lead generation process, a remarkable achievement in such a short time.

Traditional ligand binding assays in combination with molecular modelling are important tools for structure activity relationship (SAR) studies, but functional assays are required to determine ligand efficacy. The functional cellular assays that have become available recently to study ion channels show great potential in terms of screening throughput and delivering quality data to determine both the pharmacological properties of ion channel ligands and perform SAR studies. Combining the efficacy data obtained in functional assays with the affinity data obtained in ligand binding assays, ion channel pharmacophore models can now be built with much better predictability. Furthermore, the number of compounds used to build the models can be increased significantly making the SAR studies much more valuable.

Current electrophysiology platforms reach a maximal throughput of 1000-2000 data-points per day. These numbers contrast with the golden standard for an HTS assay of ~ 30000 data-points per day. The development of a truly HTS-compatible electrophysiology platform would most likely facilitate the discovery of ion channel drugs further. Although this may not occur within a 3-5 year time frame, it is already clear that a historically difficult target class “ion channels” will take front row seats in modern drug discovery programs. This has been made possible by HTS technologies like AAS flux assays and FLIPR in combination with a continuous steady flow of improved automated electrophysiology technologies for HTS-follow-up activities.

The work in this thesis demonstrates the validity of multiple methods and technologies when studying ion channel pharmacology. These developments are still in their infancy and therefore have not resulted in novel ion channel drugs yet, but within the next 5-7 years it will hopefully lead to new improved drugs especially for the treatment of chronic pain.
6 ACKNOWLEDGEMENTS

This has been a long journey considering I started to think about doing a PhD back in the early 1990s, but as the saying goes “all good things come to those who wait”. As evident from paper I and II, they were done at Sct. Hans Hospital, Department of Biological Psychiatry in Denmark and I have been privileged to have had the opportunity of obtaining my PhD while working at AstraZeneca R&D in Södertälje, Department of Molecular Pharmacology, where the work in Paper-III and IV was carried out.

I would like to express my sincere gratitude to all the people who made this possible:

**Bertil B Fredholm**, my main supervisor and **Olof Larsson**, my co-supervisor. Thanks to your efforts this endeavour was made possible. I am grateful that you both invested your time in me in spite of the special circumstances and working more like mentors than supervisors. Bertil, I have really enjoyed learning pharmacology from a true master and I’m amazed by the ability you have to correct and improve manuscripts. Olof, thank you for the never ending optimism and support even when having lots of other commitments. Thanks for sharing your vast knowledge in ion channels and your fantastic ability to make things happen.

**Åsa Malmberg**, Department Head, Molecular Pharmacology AstraZeneca R&D Södertalje. Without your support this would never have been possible. When you employed me back in 2002 this was never part of the deal and I will always be grateful that you gave me this possibility and for believing in me. I’m impressed with your ability to keep track of scientific details and always being able to pinpoint the critical issue, while successfully leading a department of 80 people. I hope to be able to pay back to the department by delivering some great ion channel assays.

**Samuel Svensson**, Section Head at Molecular Pharmacology AstraZeneca R&D Södertalje. Thanks for using your Linköpings University connections and helping me with my master’s thesis, which was required to get sufficient credentials to start at KI. Thanks for your support and comments on this thesis.

From my 10 years at Sct. Hans Hospital, Department of Biological Psychiatry in Denmark I’m eternally grateful to **Mogens Nielsen** and **Michael-Robin Witt**, thank you for the most memorable time in my life. Mogens with your passion and open mindedness you planted the interest in neuroscience in me and taught me everything I know about GABA-A receptors. Thanks for guiding me through my first publications, it is a bit like love - one never forgets the first one (the furanocoumarin story 1996). Mogens and Robin together you created a fantastic environment in the lab overlooking Roskilde Fjord, without you, science would never have meant as much to me as it does today. Robin for being a fantastic inspiration and my best friend over the years, thanks for countless hours of science, video-microscopy, patch clamping, wind surfing, bicycling, football, tennis, golf, pool, videos with chilli con carne and Coronas, but most importantly, interesting conversations. Many thanks for the numerous spelling
and grammatical corrections you have done in my publications over the years - not the least this thesis. You have always played an important role at crucial times in my life - in short, thanks for always being my wingman.

Many thanks to Tommy Liljefors, Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen and Olov Sterner, Department of Chemistry at Lund University. Tommy you were a big part of Paper-I and II and without your incredible insights in medicinal chemistry it would not have been possible. Olov thanks a lot for the collaboration over several years and many great times with a large amounts of Danish beers. Thanks for your constant supply of great chemistry PhD students who wanted to obtain some biological knowledge, it was a fun time.

To all my co-authors over the years, especially Pia Kahnberg, for a great collaboration on Paper-I. Line Hartvig, for a superb job on Paper-II and a whole lot of “we can do it” attitude during the process. Shephali Triverdi, for all the hard work on Paper-III, sending back and forth manuscripts over the atlantic. Michael Dabrowski, for all the input on Paper-IV and, as the only other Dane in the department preventing me from becoming too Swedish.

To all the great colleagues at Molecular Pharmacology AstraZeneca R&D Södertalje, in particular the ion channel crew; Eva Nyman, Urban Karlsson, Per-Eric Lund, Therese Lundström, Lucy Horoszok, Jenny Bernström, Elisabet Venyike, Jennie Strindlund, and Gunilla Brännström, thanks for making Molecular Pharmacology a fantastic working environment, you make it fun going to work every day. Many thanks and much appreciation to my current and former section heads Johannes Krupp and Robert Kronqvist for being both understanding and demanding, when managing me (not an easy assignment) in a productive way, when I was both at AstraZeneca and Karolinska. Thanks to my AstraZeneca friends who make me think of other things than ion channels Santiago Parpal, Tomas Borgegård, Fredrik Jeppsson and Johan Nord, and not forgetting the whole football team, Discovery United.

From the Karolinska Institutet, Department of Physiology and Pharmacology I would like to thank the Journal Club members for some interesting discussions. Special thanks to my fellow PhD students (now Doctors) Stina Johansson and Olga Björklund you showed me the way.

To my beloved family, Mom and Dad (Esther and George Dekermendjian) and all my little sisters Séta, Ania and Mia, thanks for your patience with me as a big brother. Mom and Dad thanks for all your love and support and for giving me the courage to believe in myself also if I wanted to do things my own way.

Finally thanks to the love of my life, my wife Tine Dekermendjian. Without you this whole endeavour wouldn’t really matter much. You are the most courageous person I know, without a hesitation you moved with me around the world and I will always admire you for that. Thanks for always being there and bringing sunshine in my life.
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