Sensing transport: label-free in vitro assays as an atTRACTive alternative for solute carrier transporter drug discovery

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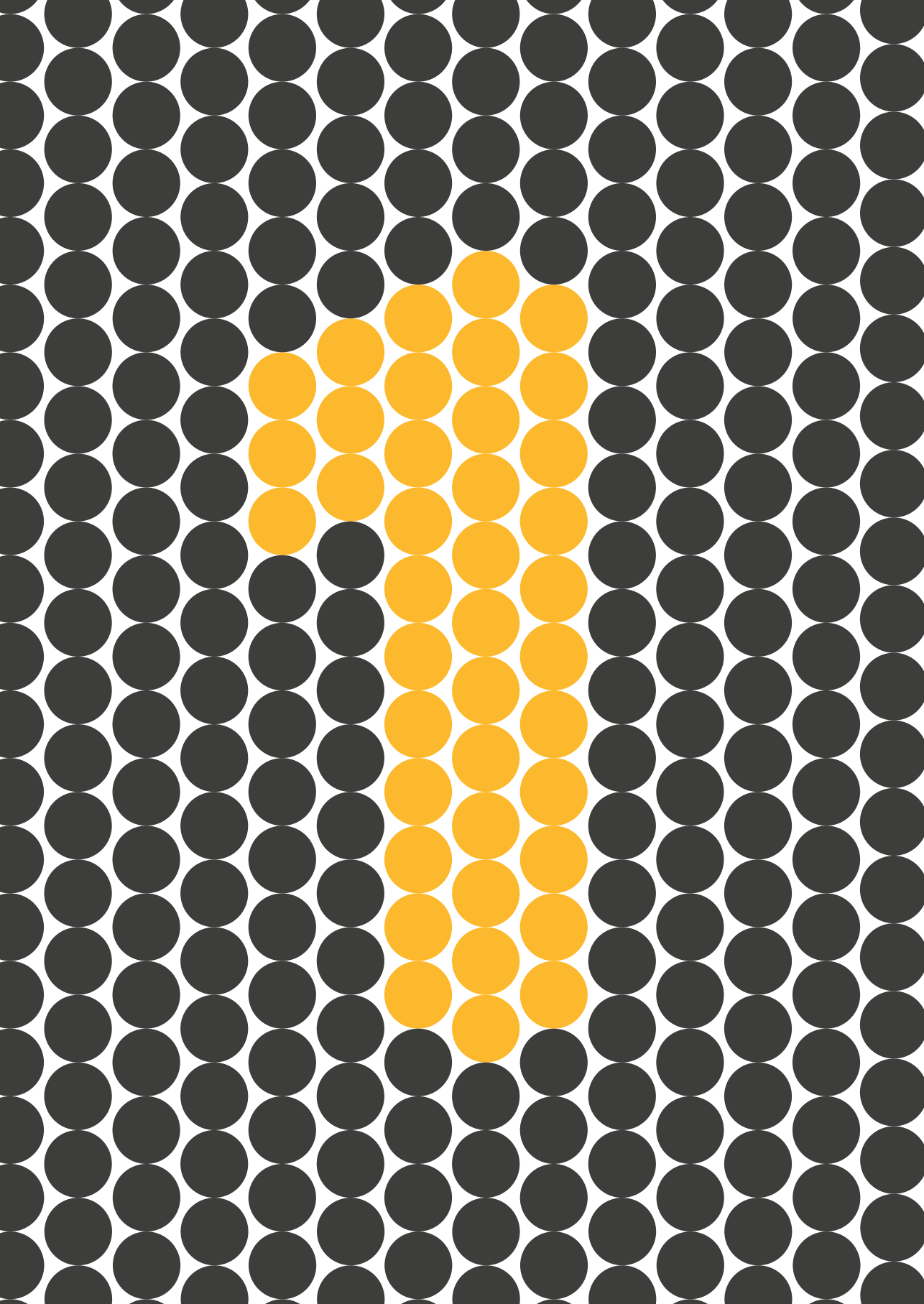


CHAPTER 1

General introduction

Solute carrier (SLC) transporters are a large and diverse class of relatively understudied transmembrane proteins. Due to their critical role in cellular homeostasis, physiological processes and disease development, there is a great number of SLCs that have the potential to be viable drug targets for the treatment of disease. Robust assays are required to identify and characterize potential drugs for SLCs, which are often screened *in vitro* using cell-based or cell-free systems. Conventional assays either require the use of chemical labels, which i) can be invasive and compromise a cell's physiology, ii) are based on end-point measurements, iii) use cell preparations and/or iv) do not allow screening of a large number of compounds. This thesis presents the development and application of novel label-free assays based on electrical impedance that allow the assessment of functional activity for three human SLCs: the dopamine transporter (DAT, SLC6A3), norepinephrine transporter (NET, SLC6A2) and excitatory amino acid transporters (EAAT, SLC1 family). With the ability to screen and characterize SLC inhibitors, these assays are a new addition to the ever-expanding toolbox for SLC transporters and could prove valuable in drug discovery programs for a wide range of diseases.

1.1 – The cell membrane, receptors and transporters

Life exists by the presence of barriers. Take any organism and its existence is legitimized by the encapsulation of its vital contents with a bilayer of lipids. This membrane demarcates the perimeter of what we define as a 'cell' and it is the true boundary that prevents the building blocks of life – the proteins, enzymes, organelles, genetic material and all else – from floating around purposelessly[1]. As such, the membrane enforces the exact organization of a cell by keeping its contents all in one place, so that the proteins and nucleic acids that makes any single cell perform its genetically imprinted function have the opportunity to interact with each other and fulfill their role. In addition, it safeguards the cell from hostile intrusions by fending off viruses, bacteria and other micro-organisms. However, the membrane is not a mere wall that hermetically shuts off the outside from the inside. On the contrary, what makes cellular membranes truly unique – and in that sense, essential to grant the existence of life – is their ability to embed specific proteins that provide cells with a means to take up nutrients, excrete waste and allow selective communication of the cell with its environment and *vice versa*[2]. By studying the molecular functions, tertiary structures, protein interactions, expression patterns and regulatory mechanisms of these proteins we obtain a better understanding of their roles in physiology (e.g., metabolism, signaling, homeostasis) and pathology (e.g., overactivity, deficiency, disease-related mutations)[3]. By utilizing this knowledge, we can rationally design and develop better drugs and interventions that – temporarily or permanently – restore or disrupt the functions of these proteins, thereby treating disease and increasing quality of life for patients[4–6].

Besides forming a barrier between the outside and the inside of a cell, the lipid bilayer membrane also functions to form subcellular compartments such as the nucleus, endoplasmic reticulum, lysosomes and mitochondria, each harboring their own cell-specific set of proteins. Taking a closer look at these cellular membranes, two main types of transmembrane proteins can be identified: receptors and transporters. In essence, a **receptor** is any protein that can bind a specific ligand (e.g., ions, small molecules, proteins), which either leads to a functional effect inside or outside the cell or is idle. Examples of receptors are G-protein coupled receptors (GPCR), receptor tyrosine kinases, immune receptors, enzymes, nuclear receptors and cell adhesion molecules[7–11]. On the other hand, a **transporter** is a protein that facilitates the active or passive movement of substrates (e.g., water, ions, small molecules, peptides) across a membrane. Examples of transporters are (ligand-gated) ion channels, ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters[12–15]. Together, these protein classes make it possible for a cell to receive extracellular signals – from its environment, other cells, or itself – and respond accordingly. Although cells can exploit other mechanisms, such as endocytosis and exocytosis, to exchange small and large molecules with its surroundings, the transporters of the transmembrane protein pools constitute the mainline of a cell's communication. It is therefore no surprise that deficits in transporters contribute substantially to the etiology of a wide range of diseases, including genetic disorders[16,17], cancer[18,19], metabolic disease[20] and neurological disorders[21], making them attractive targets for the development of therapeutic drugs.

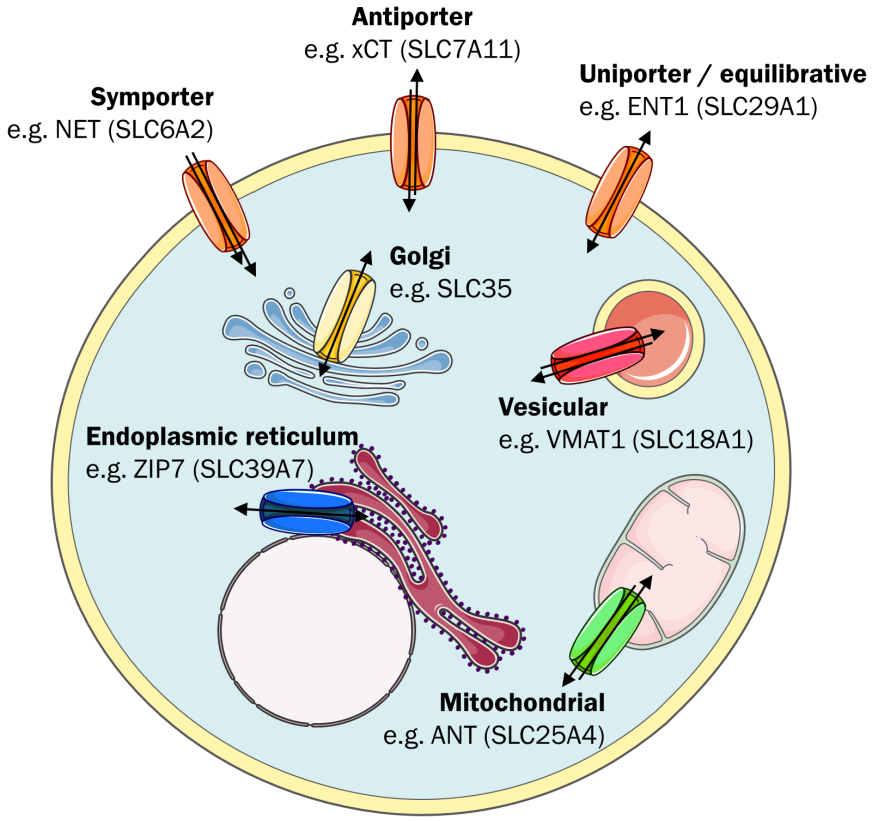


Figure 1.1 – A brief overview of the different types and subcellular localizations of solute carrier (SLC) transporters. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

1.2 – Solute carrier (SLC) transporters: an understudied group of potential drug targets

By far the largest fraction of clinically approved drugs (~30%) exert their therapeutic effect primarily or indirectly by modulation of receptors of the GPCR superfamily, where GPCRs account for 12% of all approved human drug target genes[22]. This family has a rich history of successful drug development and continues to receive widespread attention from the scientific communities[23]. However, when looking systematically at the various 'druggable' protein families versus the number of publications and the amount of drug-like compounds attributed to them, we learn there is one group in particular sticking out as one that has received relatively little attention: the **solute carrier** (**SLC**) superfamily of transporters[24,25].

SLCs comprise the second-largest membrane-bound protein family behind GPCRs, with roughly 450 members currently categorized into 66 major subfamilies and several 'atypical'

transporters being unclassified[12,14,26,27]. SLCs do not require the consumption of adenosine triphosphate (ATP) to operate and comprise both facilitative transporters – which are equilibrative and transport a substrate down its concentration gradient – and secondary active transporters – which can be antiporters or symporters that utilize an electrochemical gradient (e.g. Na^+) to transport a substrate with or against its concentration gradient (**Figure 1.1**)[12]. SLCs are mainly localized at the plasma membrane and are known to be expressed on vesicles, lysosomes, Golgi apparatus and endoplasmic reticulum, with a large subfamily (SLC25) exclusively expressed in mitochondria[27,28]. In addition, the uptake and excretion of many pharmaceutical drugs is mediated *via* SLCs, which is crucial for drug distribution but also facilitates unwanted drug-drug interactions[29,30]. To bring order to this multitude of attributes, the nomenclature of SLCs has been instated twenty years ago to harmonize the members of this superfamily, naming the genes as "SLC" followed by the family number, a letter (usually A) and the member number (e.g., SLC1A1)[31]. Since many SLCs were first characterized prior to this harmonization, the protein names are often related to their originally described function and can differ between species (e.g., SLC1A1 is known as EAAT3 (human) and EAAC1 (rodents))[32]. As such, multiple names are often linked to a single SLC, making systematic queries confusing and cumbersome.

The division of SLCs into subfamilies is mainly based on their shared tertiary protein structure ('fold'), substrate specificity, mode of action and/or subcellular localization. As opposed to kinases or GPCRs, which share distinct structural and functional motifs and binding sites between family members, SLCs are widely different from each other even within subfamilies, which results in a notorious difficulty to develop an all-encompassing, one-size-fits-all approach to study these proteins. In part owing to the challenges that have impeded the proper characterization of transporters, the majority of SLCs have been long 'neglected' from a molecular biological and drug development perspective. In fact, it is estimated that roughly 30 percent of currently identified SLCs is regarded to be 'orphan', having no verified substrate or function ascribed to them[32,33]. Moreover, SLCs were found to be the protein family with the greatest 'publication asymmetry', meaning that the vast majority of papers focused on only a few SLCs (such as the neurotransmitter (SLC6) and glucose (SLC2) transporters) and that more than 200 SLCs have less than 15 publications attributed to them[24]. Related to this, inquiries over the past ten years have indicated that less than 5% of SLCs (<20) are targeted by approved drugs for which the mechanism of action is primarily attributed to direct modulation of the SLC, with the majority of drug classes being antidepressants (reuptake inhibitors acting *via* SLC6 family neurotransmitter transporters), antihypertensives (mainly loop diuretics acting *via* SLC12 family $Na^+/K^+/Cl^-$ transporters) and, more recently, antidiabetics (inhibitors of Na^+/glucose cotransporter 2 (SGLT2) of the SLC5 family)[24,25,34,35]. While these are communally important and widely prescribed drugs, there are immense opportunities for other SLCs that are involved in the development of other diseases.

Most of the current SLC-targeting drugs were discovered before there was any knowledge on the mechanism of action or the involvement of transporters. According to Lin *et al.*, at least 84 SLCs are implicated in rare, inheritable monogenic disorders that may form the basis for the specific development of new drugs[17]. Indeed, new disease areas are being associated

with SLC malfunction and by a more rational drug design several compounds are currently undergoing clinical trials for at least ten SLCs that previously have not been the targets of drugs[6,25]. As a result of the high disease relevance and druggability potential on the one hand and systematic lack of exploration on the other, there have been community-based efforts to ignite the spark and kick-start a wide-spread, full-on frontal approach to characterize SLCs in all their facets: functional deorphanization, disease association, biochemical reagent generation, structure elucidation, assay development and drug discovery[24,36].

1.3 – The RESOLUTE consortium: enabling SLC research

In July 2018, a group of researchers from academia, including the team which I am part of, and members of the European Federation of Pharmaceutical Industries and Associations (EFPIA) kicked off the European Innovative Medicines Initiative (IMI) consortium **RESOLUTE** – Research Empowerment on Solute Carriers (www.re-solute.eu)[36]. The main aim of this consortium is to coordinate major efforts towards the generation of long-lasting research tools to study SLCs, ultimately leading to deorphanization on a large scale: linking each SLC to its corresponding substrate(s). To achieve this, RESOLUTE operates with an emphasis on publishing open-access data and facilitates the development and generation of: 1) plasmids and cell lines, which enable the knock-out or expression of a single SLC for functional assessment; 2) "omics" approaches to identify SLC substrates and protein interactions; 3) cell-based and cell-free assays to study SLC function and identify inhibitors and modulators; 4) purified protein and SLC-specific antibodies to facilitate structure determination and assay development. The data and conclusions presented in this thesis fully support the philosophy and overarching ambitions of RESOLUTE, to the extent that it will aid drug development in innovative ways and help propel the SLCs towards the long overdue status as one of the main drug target families.

1.4 – Assays and methodologies to study SLCs in vitro

Each SLC transporter has specific stoichiometries by which it translocates ions and/or solutes across the membrane with each transport cycle. In order to understand and measure the activity of a transporter upfront knowledge is required of the amount and identity of the ions and molecules that are involved. Since it is quite challenging to directly probe protein function in live humans or animals, the initial steps in the functional characterization of an SLC are the use of *in vitro* assays. These methodologies often use native or engineered cell systems that (over)express the SLC of interest compared to cells that lack this particular SLC. Depending on the substrate(s) and stoichiometry of a transporter and its localization in the cell, various methodologies can be employed to acquire information on the transport kinetics (activity, defined by the Michaelis-Menten constant K_m, and maximal transport velocity, defined by V_{max}) and, in case of SLC inhibitors, inhibitory potencies (IC_{50}, inhibitor concentration at which 50% of the substrate uptake/effect is inhibited)[25,37,38]. SLC assays are broadly based on 1) direct detection of substrate binding, uptake or efflux; 2) secondary

Table 1.1 – Characteristics, advantages and limitations of previously established *in vitro* SLC assays

Assay	Label-free	Through-put level	Live cells	Real time	+ / –	Advantages / Limitations
Substrate detection – uptake and binding assays						
Radioactive substrate uptake (or binding)	No	Low – Medium	Yes	Yes	+	Kinetic determination (K_m, V_{max}) Versatile readout for many SLCs
					–	Requires radiolabeling of substrate Radioactive handling and waste disposal Less suitable for compound screening
Fluorescent substrate uptake (or binding)	No	High	Yes	Yes	+	Increased throughput compared to radiolabels Ease of handling and detection
					–	Non-physiological modification of the (endogenous) substrate – not feasible for every SLC Interference with fluorescent or quenching compounds
Biosensors	No	Medium – High	Yes	Yes	+	High sensitivity and specificity Direct measure of substrate concentration Suitable with intracellular SLCs
					–	Sensor development is time consuming Requires modification of the cell to express the sensor
MS-based transport assay	Yes	Low	Yes	No	+	High sensitivity and specificity Wide range of substrates, including ions Measure multiple compounds per run No labeling of the substrate
					–	End-point measurements, indirect kinetics Complex data output Expensive equipment and specialist skills
Cellular thermal shift assay (CETSA)	Yes	Low – Medium	Yes	No	+	Suitable with purified protein, cell lysates and live cells Direct interaction of compound with SLC Identification of substrates and inhibitors
					–	Not all compounds will stabilize the SLC Likelihood of false-negative results End-point measurements
Secondary effects – functional assays						
Fluorescent dyes	No	High	Yes	Yes	+	Ease of handling and detection Different types of dyes for many types of functionality – adaptable to many SLCs
					–	Often requires SLCs to be electrogenic Requires step to load dyes into cells
Phenotypic assays	Yes/No	Medium – High	Yes	Yes/No	+	Most straightforward readout based on cell viability Applicable to any SLC, irrespective of localization
					–	Requires knowledge on genotype-phenotype relation Substrate redundancy could limit specificity of assay
Electrophysiology						
Patch-clamp	Yes	Very low	Yes	Yes	+	Golden standard for channels and electrogenic SLCs Kinetic determination (K_m, V_{max}) – high time resolution Ideal for mechanistic studies
					–	Requires SLCs to be electrogenic Specialist skills and equipment Single cell throughput – not suited for screening
Solid-supported membranes (SSM)	Yes	Low – Medium	No	Yes	+	Kinetic determination (K_m, V_{max}) Variation in protein source and membrane composition Increased throughput compared to patch-clamp
					–	Requires electrogenic SLCs or charged substrates Membrane preparations, lacks physiologic environment of the SLC

functional effects upon substrate uptake or efflux; 3) changes in electrical currents elicited by substrate transport. A general overview of these assays is found in **Table 1.1**.

According to an analysis of the ChEMBL database, which is manually curated and reports protein-specific bioactivity data for drug-like molecules, by far the majority of substrate uptake (>43%) and binding assays (87%) use radiolabeled compounds[38]. Historically, radioligands have been widely used to perform pharmacological experiments due to the relative ease of the detection principle and compound radiolabeling, making the technique versatile for many SLCs[39]. The assay readout is based on the amount of radioactivity bound to the SLC (in the case of binders/inhibitors) or accumulated in the cells (in the case of substrates).

However, a major concern of the use of radioactivity is that a specialized infrastructure is required for appropriately certified lab space, personnel and waste disposal, which can be costly and is not always available. Although it remains the most universal approach to assess SLCs, high-throughput screening (HTS) is less suitable with this technique. Improvements in this regard have been made using the more high-throughput scintillation proximity assays (SPA)[40]. These assays often require purified and solubilized protein which is challenging to achieve for SLCs, although whole-cell SPAs have been reported for some SLCs[41,42].

An alternative to radiolabels is the use of fluorescent compounds. In contrast to radioactivity, fluorescence does not require stringent safety precautions and can be measured with most conventional (and HTS) plate readers, which makes the ease of use of fluorescent techniques a major advantage[43]. For some SLCs, fluorescent substrate analogs are available that mimic transport properties of endogenous substrates, for example the neurotransmitter analog ASP^{+}[44] and BODIPY-conjugated fatty acid analogs[45]. The highly sensitive readouts and increased throughput – thanks to high-capacity, robotics-operated readers such as the fluorometric imaging plate readers (FLIPR)[46,47] – make these assays ideal for screening, but come at the cost of a non-endogenous substrate that could display altered kinetics and the potential interference of autofluorescent and quenching compounds. In addition, fluorescent analogs are not readily available for all SLCs, making this approach not universal.

Other techniques that probe SLC function also rely on the use of fluorescence as a readout. Biosensors and dyes, which are either genetically encoded or loaded into the cell, are proteins or complex molecules that contain a moiety that fluoresces or is quenched in the presence of a (SLC) substrate or upon changes in voltage or ionic concentrations. Expression of biosensors can be genetically steered towards various subcellular membranes, offering the advantage to assess intracellular SLCs. Biosensors are often tailor-made to detect a specific substrate[48,49], which requires thorough optimization for each application and making them less generally applicable, but the advantage is that they can be used *in vivo*[50]. Fluorescent dyes, such as membrane potential dyes or ion-specific dyes, measure indirect events following substrate transport[44,51]. As such, these dyes are used for functional assays that can be applied to a wide range of SLCs, given that they induce these secondary effects upon substrate transport. As is the case with other label-based techniques, the loading of the fluorescent dyes could compromise the cellular physiology, making the system more artificial.

The downsides that radioactive or fluorescent labels bring along in SLC assays can be overcome by using label-free techniques, which have been gaining more traction in recent years. Liquid chromatography (LC) and mass spectrometry (MS) are increasingly employed to analyze samples of substrate-treated SLC-expressing cells – both its intracellular and extracellular contents. By quantifying the intracellular accumulation of a substrate after a given amount of time, this provides a direct measure of SLC activity, i.e. influx and efflux of substrate are indirectly detected[52]. Alternatively, LC-MS can be used for targeted and untargeted metabolomics, which allow the quantification of a selected set of metabolites (targeted)[53,54] or the identification of unknown substrates from plasma or medium samples (untargeted)[55,56] upon perturbation in cells expressing the transporter. These approaches, when applied successfully, give insight in the mechanism of (orphan) transporters and their role in metabolism[56]. However, the highly advanced equipment and complex data output might make this approach less suited for screening, although cost-effective improvements such as matrix-assisted laser desorption/ionization (MALDI) MS-based approaches rapidly follow up each other[52].

When the identification of SLC binders (i.e., substrates or inhibitors) is of importance, but the determination of uptake kinetics is not of interest, then other types of assays can be employed. Thermal shift assays (TSA) are based on the thermostabilization of a purified protein in the presence of a molecule that binds – and thereby stabilizes – the protein[57]. The cellular thermal shift assay (CETSA) detects chemical engagement between ligands and membrane proteins on cell lysates or whole cells, which has been successfully validated for SLCs[58]. In addition, the possibilities to use CETSA for screening purposes are expanding with the use of green fluorescent protein (GFP)-tagged SLCs[59]. However, in all cases CETSA uses end-point measurements and is prone to false-negative hits if a compound does bind, but does not stabilize the protein. At the same time, since not every ligand stabilizes a protein upon binding, this makes the assay relatively resistant to false-positive hits[60].

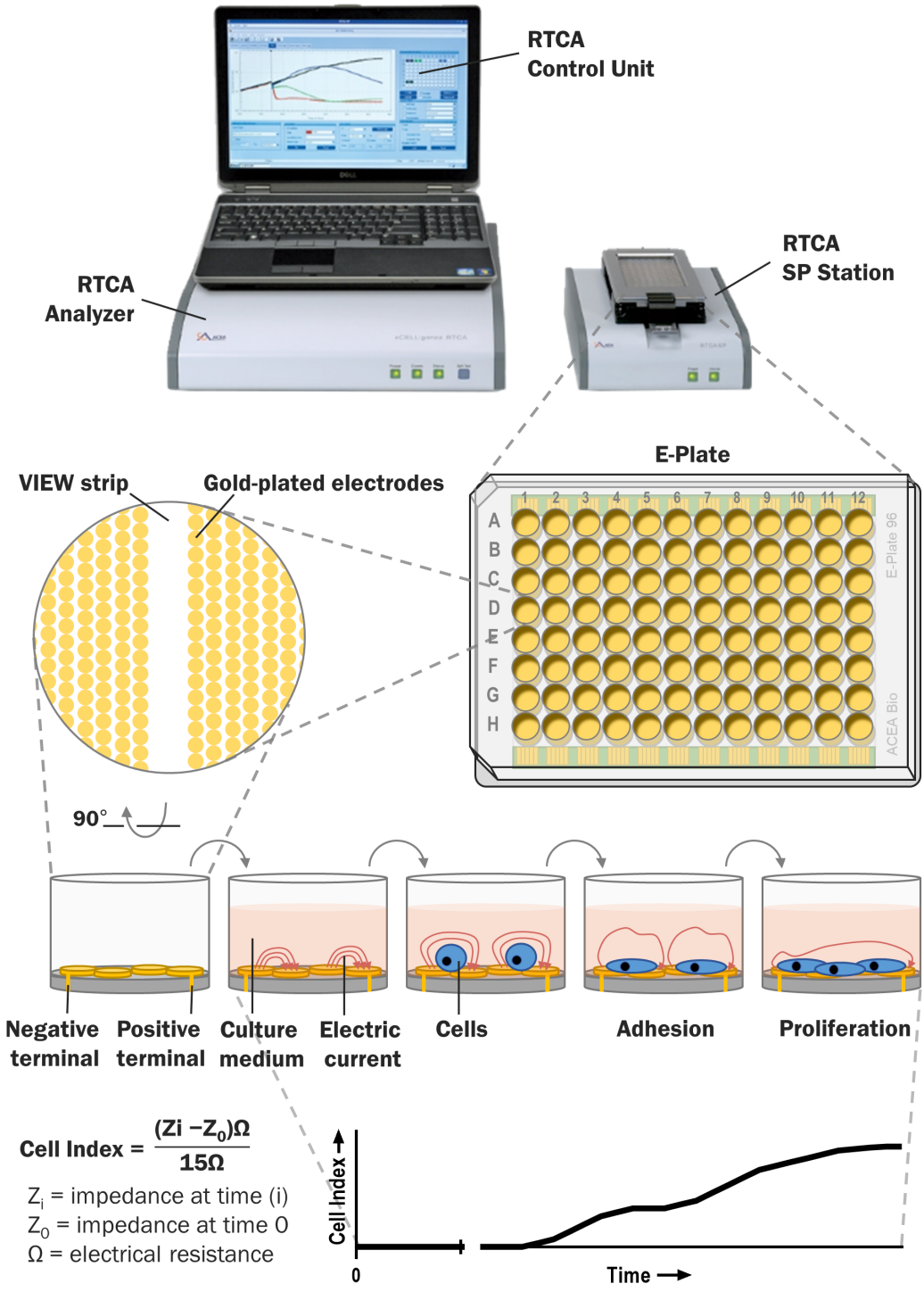
A more general approach to assess the role of a protein in a specific functional outcome is a phenotypic assay. Here, any substrate-induced downstream physiological response can be used as a readout, such as cell viability, gene expression, adhesion or differentiation. While a phenotypic assay is in general less specific and prone to false-positive results – e.g., cell death can be induced *via* multiple pathways – it can usually be performed in a high-throughput setting, making it more suitable for screening purposes[61]. For example, these approaches can be interesting to identify the uptake of cytotoxic drugs, by expressing and knocking-out one or multiple sets of SLCs in a model cell line[62]. In this sense, such assays are of tremendous use in determining the mechanism of action and in identifying inhibitors of an SLC. Despite its straightforward readout, the assay requires a robust establishment of the relationship between SLC function and the phenotypic response, which will be unique for each substrate and SLC. Alternatively, SLCs with undefined substrates could be screened for the transport of specific substrates that elicit a phenotypic response, aiding in the deorphanization process.

The aforementioned assays are either based on the use of labels or, if this is not the case, do not permit real-time measurements of the transport processes. A traditional technique to investigate transporters in a label-free setting is the use of patch-clamp electrophysiology, in which the rapid transporter-mediated electrical currents of a single cell are continuously detected in real-time *via* carefully applied electrodes[63]. This method provides unmatched high-quality data on ions flowing through ion channels or SLCs, and as such is ideal for mechanistic studies[64]. However, the laborious procedure, performed with skilled personnel and specialized equipment, makes this approach unsuitable for screening large numbers of compounds. Automated electrophysiology has greatly improved the handling and throughput, but the signal-to-noise ratio is too low to detect the small currents which SLC transporters usually generate.

In response to this, the arrival of the surface electrogenic event reader (SURFE^{2}R) enabled cell-free electrophysiology of proteins in cell membrane fragments, opening up the possibility to screen large numbers of compounds[65]. The technique is based on the use of solid supported membranes (SSM), to which membrane vesicles containing the transporter are adsorbed. Electrogenic substrate transport into the vesicles leads to a charge difference across the membrane, which generates a capacitive current *via* gold-plated electrodes that is monitored in real-time. Although the SSM-based method has been successfully applied to a range of SLCs[66], it requires the SLC to be electrogenic (i.e., net positive or negative charge per transport cycle) and is only operable with membrane fractions or artificial liposomes, meaning that this assay cannot be performed in live cells with the appropriate, physiologically relevant environment.

This extensive toolbox of assays that are available for SLCs demonstrates that over the past years innovative ways have been found to investigate transport activity, most of which have proven invaluable for the identification of substrates, inhibitors or modulators. However, considering the various methodologies in **Table 1.1**, it is striking that there is no single assay that ticks all of the boxes. As such, there seems to be an opportunity for a method that is both label-free, compatible with high throughput screening, uses live cells and allows real-time measurements. By making rational use of techniques that have been developed over the past twenty years, this thesis presents the development and application of a novel method that aims to improve upon the limitations of the more conventional assays.

→ **Figure 1.2** – Overview of the xCELLigence real-time cell analysis (RTCA) system. The example shows a 96-well E-plate, which is compatible with a Single Plate (SP) station, but the same principles apply to 16- and 384-well plates. The wells of the E-plate are covered with gold-plated electrodes. The VIEW strip at the bottom of each well allows the user to inspect the E-plate under a light microscope. An electric current is applied to the electrodes in the presence of a conductive medium. The presence, adhesion and proliferation of cells on the electrodes increases the impedance, which is a form of electrical resistance. Impedance is converted to the dimensionless parameter Cell Index, which is plotted in real-time.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Analyzer
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ACEA Bio
90°
Negative terminal
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Electric current
Cells
Adhesion
Proliferation
Cell Index =
(Zi −Z0)Ω
15Ω
Zi = impedance at time (i)
Z0 = impedance at time 0
Ω = electrical resistance
Cell Index
0
Time

1.5 – Label-free impedance-based biosensors: an opportunity for SLC drug discovery

To circumvent the use of chemical labels for pharmacological studies, several label-free techniques have been developed, some of which have been used to study SLCs and are mentioned in the previous section. However, if we want to interrogate a protein in its physiological environment, it is crucial to be able to perform the experiments on live cells with a relevant genetic background (e.g., disease-relevant cell lines). Thus, cell-based label-free assays offer the possibility to study target pharmacology while monitoring live cell behavior at physiological temperatures and in appropriate culture conditions.

In such settings, cells and proteins 'behave' more closely to how they would in the human body and provide a better representation of their native physiology. As such, cell-based label-free assays could aid in the translation of *in vitro* data towards *in vivo* outcomes, which would lead to the advancement of effective therapeutics in an earlier stage of drug development. Since these assays focus on changes in whole cell properties – adhesion, proliferation, migration, morphology – they are considered to be phenotypic assays. Techniques that are used to detect this live cell behavior mainly use optical or electrical sensors to generate a readout[67]. Optical biosensors use the refractive properties of light to detect changes in cell shape or protein conformation, examples of which are surface plasmon resonance (SPR), resonant waveguide grating (RWG) and dynamic mass redistribution (DMR)[68]. These techniques will not be further discussed, as these were not used in the context of this thesis.

Electrical biosensors are mostly based on **impedance** – a form of electrical resistance – and make use of highly capacitive (usually gold-plated) electrodes onto which cells can grow allowing their behavior to be monitored in real-time[69]. In a pioneering study from 1984 Giaever & Keese used electrical cell-substrate impedance-sensing (ECIS) with a single electrode to infer the movement of cultured fibroblasts[70]. Since then the low throughput of this system prevented it from widespread use in the screening of compounds, which ultimately led to the development of impedance-based biosensors with increased throughput such as CellKey (discontinued) and **xCELLigence** (**Figure 1.2**)[71]. Impedance-based biosensors have since been used as a versatile *in vitro* tool to study cellular properties such as adhesion[72], viability[73], proliferation[74], migration[74] and contractility[75], as well as monitoring compound cytotoxicity[75,76] and functional activity of receptor tyrosine kinases[77] and, mostly, GPCRs[78,79]. Thanks to its broad range of possibilities, the xCELLigence was selected for the development of SLC assays and will be the prevalent technology in the chapters of this thesis.

The xCELLigence, which is often referred to as a real-time cell analyzer (RTCA), consists of a central control unit – a laptop that collects and visualizes the processed data in real-time – connected to an analyzer that receives its input from a recording station, which is a device located inside a regular cell culture incubator that holds the microtiter plates – called E-plates – onto which the cells are grown (**Figure 1.2**). Depending on the type of recording station, E-plates can contain 16, 96 or 384 wells, which makes the platform amenable to HTS applications[80]. At the bottom of each well is an interleaved array of gold-plated electrodes

connected to a positive and negative terminal and in the presence of a conductive fluid (e.g., buffer or cell culture medium) a weak electrical signal can be applied. This signal is generated by voltages in the mV range with the resulting currents being in the µA range, which are non-invasive for cells allowing repeated measurements during an experiment. The voltages are applied at three predetermined, midrange frequencies – 10, 25 and 50 kHz, of which 10 kHz is the most standard[81] and is used for analysis in this thesis – which produce transcellular currents that grant the detection of changes in cell density and morphology[82]. The electrical current allows the measurement of impedance – that is a combination of the electrical resistance of the solution and the impedance at the electrode surface – which can be consecutively measured by the recording station at intervals of several seconds to minutes. The analyzer receives the impedance values and converts it to a unitless parameter

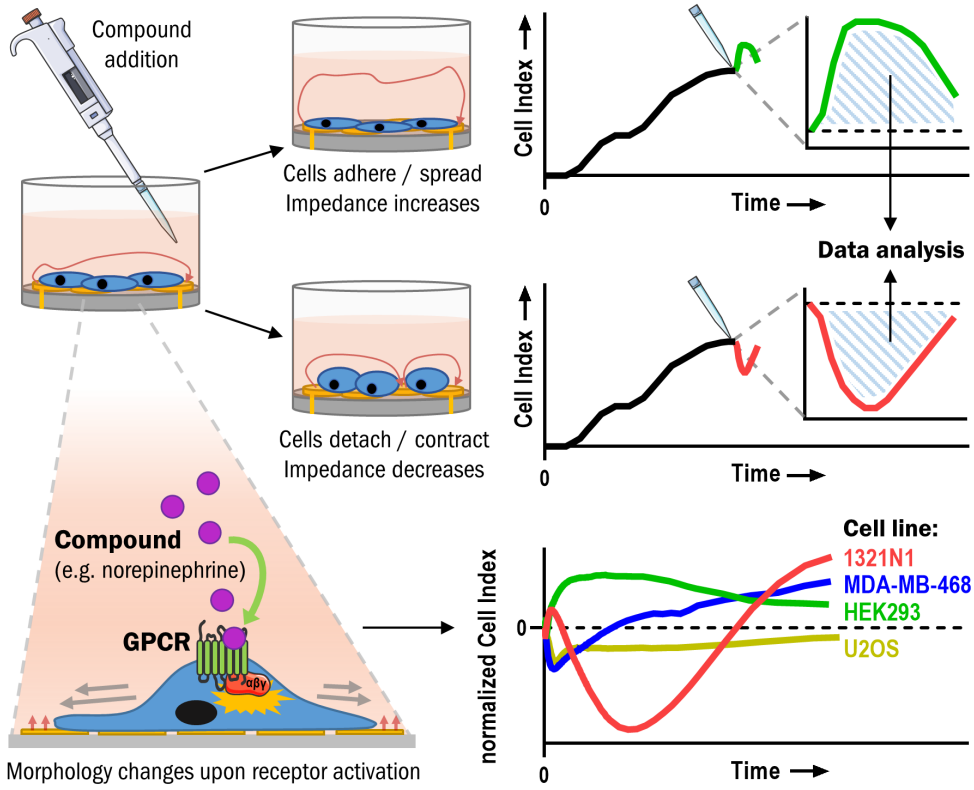

Figure 1.3 – Stimulation of cells with a compound of interest (e.g. a GPCR agonist) results in the engagement of secondary messengers (e.g., G proteins) and subsequent changes in cell morphology that are recorded as a characteristic change in impedance (i.e., Cell Index) over time. The resulting cellular response is dependent on the compound that is used, the subtype of receptor(s) expressed and the cellular background. The bottom-right illustration demonstrates that a single compound (in this case, norepinephrine) produces four different cellular responses over time in four different cell lines. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

called Cell Index (CI) which is then graphed as a function of time (**Figure 1.2**). In the presence of cells, the CI at the electrode surface increases as the cells start to settle and adhere to the bottom of the well. When cells start to spread and proliferate, the CI will generally increase. When cells start to shrink and detach, the CI will generally decrease. This way, the CI is suggestive of cell number and morphology, which can be used to substantiate any hypothesis on cellular functions.

A major application of impedance-based biosensors is their use as a functional assay for GPCRs[78,79]. A GPCR is a receptor – usually located at the plasma membrane – consisting of seven transmembrane α-helices that upon activation by a ligand (e.g., neurotransmitters, hormones, drugs) couples to intracellular G proteins and other secondary messengers that trigger cascades of downstream processes[83]. Activation of specific heterotrimeric G proteins can generally lead to increased (G_s) or decreased (G_i) production of cyclic adenosine monophosphate (cAMP), increased Ca^{2+} levels (G_q) and activation of Rho GTPases ($G_{12/13}$), which can regulate a myriad of cellular processes including gene transcription, proliferation, cell contractility and actin cytoskeleton remodeling[84,85]. Since each GPCR is favorably linked one or more secondary messengers, biochemical assays are used to study each pathway individually as a functional measure of GPCR activation by agonists and/or inhibition by antagonists[86]. However, multiple assays would be needed to capture the entire functional profile of the GPCR, which can be costly and time-consuming. To this end, impedance-based assays have been employed to detect changes in cell morphology, resulting from upstream GPCR activation, as a change in impedance – defined as a cellular response – which can be used to assess receptor pharmacology in live cells (**Figure 1.3**)[78,79,87].

Impedance measurements detect the sum of all downstream cellular events that follow upon GPCR activation, which results in a characteristic change of the CI over time that is dependent on the ligand, receptor subtype, the pool of intracellular secondary messengers and the cellular background[85] (for a comprehensive overview of response profile of GPCRs in impedance assays, see Doijen *et al.*[81]). By titrating the amount of ligand in different wells, potency values (EC_{50}, the concentration at which a compounds induces 50% of the maximal effect) can be determined for ligands that engage with a specific receptor, which are often comparable to those obtained in other functional assays[88–90]. To this end, cell lines with heterologous overexpression are usually used to ensure receptor specificity, a high number of receptors on the cell surface and – ideally – a better signal-to-noise ratio. However, due to the high sensitivity of the electrical sensor, it is also possible to detect cellular responses in cells with endogenous expression of the GPCR[91,92] and in non-adherent cells by using a plate coating[93], which provides a readout that is more physiologically relevant than heterologous expression systems. This vastly expands the potential applications of xCELLigence and offers advantages over other biochemical assays that are less sensitive.

So far, very little studies have made use of cell-based label-free assays to primarily investigate SLC function. There is a single paper that reports an optical DMR assay (using the EPIC technology) for the sodium-phosphate cotransporter 2b (NaPi-2b, SLC34A2) where the addition of inorganic phosphate to overexpressing MDCK cells results in an increased DMR response, although no mechanistic explanations are provided[94]. In other instances

the xCELLigence was used to detect cytotoxicity induced by the uptake of bacterial cyclopeptides *via* organic anion transporting peptides (OATP1A2, 1B1 and 1B3, SLCO family) in pancreatic cancer cells[95], as well as cell adhesion which was enhanced in corneal endothelial cells overexpressing a sodium-bicarbonate transporter (NaBC1, SLC4A11)[96]. However, the true potential of xCELLigence as a functional assay for SLCs had thus far not been unlocked.

Recently, a pioneering xCELLigence study by our lab (Vlachdimou *et al.*) demonstrated that cellular responses upon activation of endogenous adenosine receptors (ARs) in an osteosarcoma cell line (U2OS) were diminished in the presence of the endogenous equilibrative nucleoside transporter 1 (ENT1, SLC29A1) – a transporter of the adenosine – on the same cells[97]. Pharmacological inhibition of ENT1 restored the activation of ARs, which the authors defined as a measure of transporter function that could be used as an assay to identify binding and kinetics of ENT1 inhibitors[98]. Thus, these data demonstrate a novel application of impedance-based biosensors to study SLC activity in live cells without the use of chemical labels. Based on these initial findings, it is expected that this label-free assay – which is termed the 'transport activity through receptor activation' (**TRACT**) assay in this thesis (**Figure 1.4**) – is not exclusive to the adenosine system, but is more widely applicable to other SLCs as well.

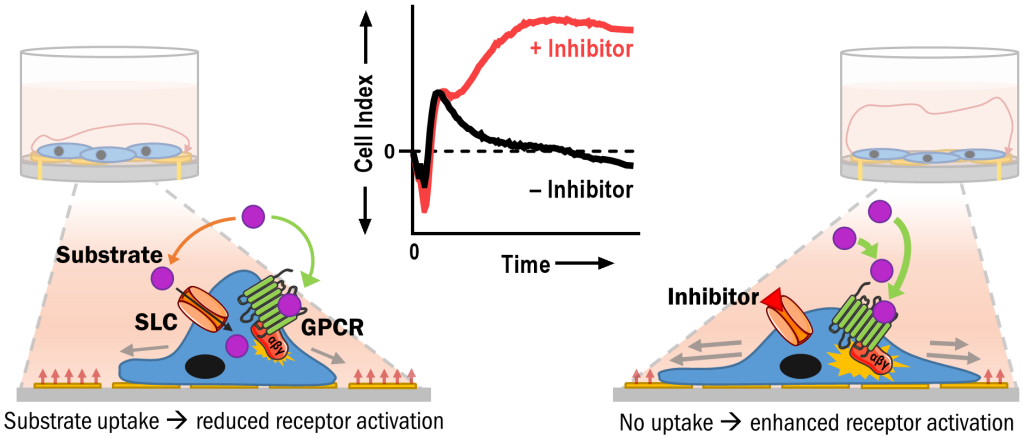

Figure 1.4 – Visualization of the 'transport activity through receptor activation' (TRACT) assay principle on xCELLigence. In cells that express both an SLC and GPCR that recognize the same substrate, addition of this substrate to the medium will lead to its uptake *via* the SLC, resulting in a reduced extracellular concentration of substrate at the cell membrane and, simultaneously, reduced activation of the GPCR which is detected as a change in impedance (i.e., Cell Index) over time. In the presence of an SLC inhibitor, the substrate will not be taken up, resulting in a relatively elevated extracellular concentration of substrate at the cell membrane. As a result, a larger fraction of the substrate is available to activate the pool of GPCRs, resulting in more drastic changes in cell morphology and Cell Index compared to the cells that did not receive the inhibitor. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

1.6 – Aim and outline of this thesis

AIM

The main aim of this thesis is to explore the possibilities of the label-free impedance-based biosensor xCELLigence to study the pharmacology of selected SLCs, with the purpose to provide novel compound screening tools for drug discovery to the transporter communities and beyond. The assay principles that were described in the study by Vlachodimou *et al.*[97] were used as a starting point for the initial selection of amenable SLCs: if an SLC and GPCR, which respectively transport and are activated by the same substrate(s), are expressed on the plasma membrane of same cell, then the SLC will directly affect the extracellular concentration of the substrate(s) and thereby affect the magnitude of GPCR activation by the substrate(s). Thus, at the base of assay development is the identification of an SLC–GPCR 'pair'. When looking at the number of known SLCs, GPCRs and endogenous substrates, besides the aforementioned couple of ENT1–ARs and adenosine, there are many more of such 'pairs' that play a role in physiology. An extensive overview of these can be found in the **Appendix** (**Table A.1**), which will be further discussed in **Chapter 8** of this thesis.

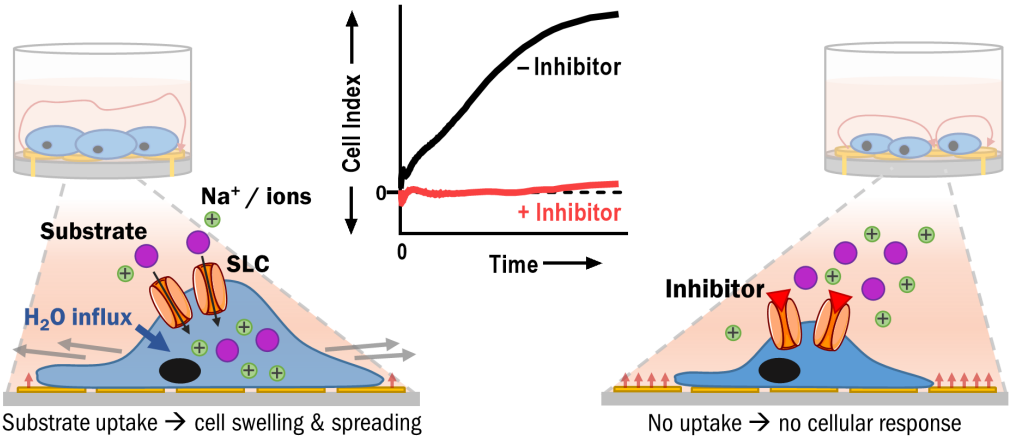

Figure 1.5 – Visualization of the impedance-based phenotypic assay based on SLC-mediated cell swelling. In cells that express an SLC that mediates uptake of a substrate and/or ions, addition of this substrate to the medium leads to influx of substrate and ions. Accumulation of these ions in the cytosol generates an osmotic gradient over the cell membrane. This causes the influx of water and regulation of the ionic content by ion channels and transporters. The resulting increase in cell volume triggers secondary events that lead to cell spreading, which increases the electrode coverage and, thus, the impedance (i.e., Cell Index). SLC inhibition prevents these cellular responses upon addition of the substrate. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

OUTLINE

In **Chapter 2**, a short overview is given on the most recently described examples of SLCs that modulate the activation of GPCRs, which expand upon the traditional dogma of SLCs that remove extracellular substrate from the receptor compartment.

Chapter 3 describes the development of a TRACT assay (**Figure 1.4**) for the human dopamine transporter (DAT, SLC6A3) on the xCELLigence, which is an extension of the previously described assay for ENT1[97] and is demonstrated in two cell lines each with endogenous expression of a dopamine-responsive GPCR and heterologous expression of DAT.

Chapter 4 reports a label-free TRACT assay for the human norepinephrine transporter (NET, SLC6A2), which expands upon the previous chapter by using various substrates, testing the assay suitability with HTS and comparing inhibitors with a fluorescent substrate uptake assay.

Chapter 5 describes the application of the TRACT assay to test the inhibitory activities of compounds that were predicted by virtual screening to be inhibitors for NET, which demonstrates the applicability of the TRACT assay in a drug discovery setting.

In **Chapter 6**, the xCELLigence is used to detect the activity of excitatory amino acid transporters (EAAT, SLC1 family) using a TRACT assay and a novel impedance-based phenotypic assay based on EAAT-mediated changes in cell morphology as a result of cell swelling (**Figure 1.5**).

Chapter 7 describes the application of the phenotypic assay to characterize substrate and inhibitor responses on ten missense mutants of EAAT1 that were found in cancer patients and rare cases of episodic ataxia.

Chapter 8 concludes this thesis with an overall conclusion of the various impedance-based assays that were developed and discusses their advantages and limitations. Moreover, a mechanistic substantiation of the TRACT assays in this thesis is provided by means of previously reported models. Ultimately, a call is made to increase the awareness of the primary or confounding roles of SLCs in physiological and pharmacological studies, with an emphasis on GPCR ligands, and perspectives are provided that speculate on the future of label-free assays for SLC drug discovery.

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