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**Development of an Analytical Tool to
Characterize the Dynamics of Biological
Systems Using Electrophysiological Traces**

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Resumen

Las respuestas celulares a estímulos críticos para su función están organizadas en forma de redes que integran múltiples procesos más sencillos. Las técnicas experimentales actuales únicamente nos permiten visualizar y medir estos procesos a nivel molecular en algunos casos extremadamente favorables. Por tanto, y para el avance de nuestro entendimiento de las respuestas fisiológicas, es crucial el desarrollo de herramientas y métodos que nos permitan extraer a partir de la respuesta celular global (macroscópica) que podemos medir, la dinámica de los distintos procesos a nivel molecular (microscópicos) así como la relación entre los mismos. En esta tesis se presenta el desarrollo de un método híbrido computacional/analítico para realizar dicha tarea: la herramienta SYSMOLE (SYStems-based MOLeclar kinetic scheme Extractor). SYSMOLE utiliza teoría de identificación de sistemas con el fin de obtener una función de transferencia entre el estímulo (entrada) y la respuesta global de la célula (salida) en el dominio transformado de Laplace. Una vez caracterizada dicha función de transferencia, SYSMOLE obtiene una cadena de Markov o modelo cinético molecular asociado a la misma utilizando un proceso de clasificación e imponiendo ciertas restricciones biológicas para condicionar el problema. Primero utilizamos trazas sintéticas para evaluar las prestaciones de SYSMOLE en términos de velocidad de convergencia, obtención del modelo cinético molecular correcto, y robustez frente al ruido. Después examinamos el funcionamiento de SYSMOLE en su aplicación al análisis de trazas electrofisiológicas de canales de calcio activados durante la despolarización de la membrana, y mostramos que SYSMOLE no sólo obtiene el modelo cinético molecular que describe la activación e inactivación de dichos canales, sino que también identifica correctamente el mecanismo de acción de la nifedipina, un bloqueador de canales de calcio utilizado clínicamente para tratar enfermedades cardiovasculares. Finalmente, presentamos la aplicación de SYSMOLE al estudio de la farmacología y señalización de una nueva clase de fármacos antipsicóticos cuya diana es un complejo heteromérico de receptores acoplados a proteínas G. Los resultados indican que la herramienta desarrollada en esta tesis es capaz de obtener modelos cinéticos moleculares relevantes a partir de trazas electrofisiológicas y que puede ser de utilidad para el estudio de una amplia gama de sistemas biológicos.

Abstract

Overall cellular responses to biologically-relevant stimuli are mediated by networks of simpler lower-level processes. Although information about some of these processes can now be obtained by visualizing and recording events at the molecular level, this is still only possible in especially favorable cases. Therefore the development of methods to extract the dynamics and relationships between the different lower-level (microscopic) processes from the overall (macroscopic) response remains a crucial challenge in the understanding of many aspects of physiology. In this thesis we describe the development of a hybrid computational-analytical method to accomplish this task, the SYStems-based MOLeclar kinetic scheme Extractor (SYSMOLE). SYSMOLE utilizes system-identification input-output analysis to obtain a transfer function between the stimulus and the overall cellular response in the Laplace-transformed domain. It then derives a Markov-chain state molecular kinetic scheme uniquely associated with the transfer function by means of a classification procedure and an analytical step that imposes general biological constraints. We first tested SYSMOLE with synthetic data and evaluated its performance in terms of its rate of convergence to the correct molecular kinetic scheme and its robustness to noise. We then examined its performance on real experimental traces by analyzing macroscopic calcium-current traces elicited by membrane depolarization. SYSMOLE derived the correct, previously known molecular kinetic scheme describing the activation and inactivation of the underlying calcium channels and correctly identified the accepted mechanism of action of nifedipine, a calcium-channel blocker clinically used in patients with cardiovascular disease. Finally, we applied SYSMOLE to study the pharmacology of a new class of glutamate antipsychotic drugs and their crosstalk mechanism through a heteromeric complex of G protein-coupled receptors. Our results indicate that our methodology can be successfully applied to accurately derive molecular kinetic schemes from experimental macroscopic traces, and we anticipate that it may be useful in the study of a wide variety of biological systems.

Acronyms

2AR Serotonin Receptor 2A

2CR Serotonin Receptor 2C

ARC Accessory Radula Closer

ARX AutoRegressive with eXogenous variable

CVF Covariance Fitting

D2R Dopamine Receptor 2

DFT Discrete Fourier Transform

DHP Dihydropyridine

FIR Finite Impulse Response

GDP Guanosine Diphosphate

GIRK G protein Sensitive Potassium Channels

GTP Guanosine Triphosphate

LPS lipopolysaccharide

LSD Diethylamide

LTl Linear Time-Invariant

MAP Maximum A Posteriori

mGluR2 Metabotropic Glutamate Receptor 2

mGluR3 Metabotropic Glutamate Receptor 3

MKC Molecular Kinetic Converter

NMDA *N*-methyl-*D*-aspartate

ODEs Ordinary Differential Equations

PCP Phencyclidine

PDF Probability Density Function

PEM Prediction-Error identification Methods

SNR Signal-to-Noise Ratio

SYSMOLE SYStems-based MOLeclar kinetic scheme Extractor

TEVC Two-Electrode Voltage Clamp

VC Voltage Clamp

WGN White Gaussian Noise

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Chapter 1

Introduction

1.1 Microscopic vs. Macroscopic

In order to ensure their survival and correct physiological function, cells must respond appropriately to many different kinds of signals, such as the presence of various neurotransmitters or hormones in the extracellular fluid, the depolarization of the cell membrane, or stimuli such as mechanical force or light (1). The cell's response to any particular stimulus engages vast numbers of single molecules whose actions combine to perform various tasks within the cell.

To provide a framework for the study of such cellular responses to stimuli, biologists try to understand the actions of the single molecules in terms of elementary (microscopic) molecular processes, such as the opening or closing of ion channels in the membrane, the phosphorylation or dephosphorylation of intracellular proteins, or the nuclear translocation of transcription factors (2, 3).

Clearly, it is desirable to study these microscopic processes directly. This has been possible, to some extent, in a few areas, notably the biophysics of ion channels, an exceptionally favorable case where the exquisitely sensitive patch-clamp technique (4, 5) is able to record the activity of single ion channels. As a result, this technique provides some measure of direct information about microscopic processes such as the opening and closing of the channels and allows the construction of molecular kinetic models of these processes (6, 7).

In most areas of cell biology, however, such direct access to the micro-

scopic processes is not yet possible. Although new single-molecule techniques provide ways of understanding the properties of key biological molecules in isolation, monitoring the dynamics of single molecules inside cells is technically complex and in many experimental systems not practical (see (8, 9) for reviews).

The information that is routinely available from cells exposed to stimuli is the overall (macroscopic) cellular response. This response combines the signal from a large number of molecules and multiple microscopic processes (8, 9). The macroscopic response trace, which is relatively easy to record in most experimental systems, thus contains information about the microscopic processes. An important and elusive goal in the systems biology field has been to extract information from macroscopic responses regarding the microscopic processes underlying them as well as the way in which these processes relate to each other. This, however, constitutes a challenging problem.

1.2 Need of a Tool to Derive Microscopic Information from Macroscopic Traces

As mentioned in the previous section, a correct characterization of the dynamics of different molecular processes in the cell is fundamental for understanding normal physiological and pathophysiological responses. In most biological systems the direct study of these microscopic molecular processes is not yet possible due to experimental limitations, and only overall macroscopic cell responses to stimuli (macroscopic traces) can be obtained.

The subject of this thesis is the description and application of a method that combines computational and analytical approaches to extract information from macroscopic traces regarding the molecular microscopic processes and the way in which they are combined. This tool, the SYStems-based MOlecular kinetic scheme Extractor (SYSMOLE), conveniently describes the dynamics of these microscopic processes responsible for the response in the form of a molecular kinetic scheme analogous to those used in biophysics and pharmacology.

1.3 Thesis Organization

We first provide a description in biophysical terms of the problem that SYSMOLE aims to solve, followed by a state-of-the art review of other methods currently available to tackle similar problems (*Chapter 2*), as well as a general description of the approach based on System Identification used by SYSMOLE (*Chapter 3*). We next provide a detailed description of SYSMOLE's implementation (*Chapters 4, 5 and 6*), its robustness to noise (*Chapter 7*), and scalability (*Chapter 8*). On the last part of the thesis we examine SYSMOLE's performance with real experimental traces by deriving from macroscopic calcium-current traces the mechanism of action of the antihypertensive drug nifedipine (*Chapter 9*), and show how it can be successfully applied to study the pharmacology of heteromeric complexes of G protein-coupled receptors (*Chapter 10*) to generate new pharmacological hypotheses.

Chapter 2

State of the Art

Introduction. In this chapter we will present the classical description of molecular kinetic schemes in biophysics, and we will review current methods aimed at deriving microscopic insight from macroscopic experimental traces.

2.1 Molecular Kinetic Schemes

Molecular kinetic schemes were classically developed in biophysics, in particular in the ion channel field, and then adopted in biology at large to describe molecular dynamics and state changes.

Molecular Kinetic Schemes are hidden Markov chain models which describe the transitions between different states or conformations of a protein. Since the patch recording technique was introduced (5), it has been possible to study a variety of single ion channels. The channel conformations observed differ in their conductances, covering a range of about three orders of magnitude. A common feature of all the channels examined so far is their rapid transition among a number of discrete conductance levels, however the patterns of opening and closing of each channel are characteristically different.

A Markov process is a sequence of events for which the probability of an event depends only on the immediately preceding event. Most models of the kinetics of ion-channel gating, the process leading to a channel opening, can be modeled as Markov processes with discrete states (see (10) and (11) for exceptions). The simplest Markov process has the form described in Figure 2.1, where k_{CO} and k_{OC} are rate constants.

Figure 2.1: Simplest Markov process with an open and a closed state.

This simple model illustrates a number of features of discrete-state Markov models. They consist of a number of discrete states connected by paths with specified rates of transition from one state to another. The probability of a channel making a particular transition to another state depends only on the currently occupied state, and neither on previously occupied states nor on how long the present state has been occupied. For a Markov process in continuous time, it usually follows that the distribution of lifetimes of each state is a single decaying exponential. For the model in Figure 2.1 the probability density function for lifetimes in the closed state as a function of time is given by:

$$f_c(t) = k_{CO}e^{-k_{CO}t} \quad (2.1)$$

In general, for any channel one can determine the parameters of this probability density function from experimental data (Fig. 2.2) by plotting a histogram of the number of closed intervals of a given length. Likewise, one can plot a histogram of the number of open intervals against their duration or length. These distributions provide information about which models are appropriate to describe the channel. Generally, a single exponential curve does not fit the closed time distribution well. Assuming that all the experimental data come from a single channel, this implies that there is not just a single Markovian closed state. The usual solution to this problem is to postulate a model with a certain number of Markovian closed states with the corresponding closed-time distribution being a sum of exponential components. The closed-time histogram is then fitted to a multi-exponential curve, in which the number of exponential components for a good fit sets a minimum for the number of closed states in the model. An analogous procedure is applied to the open-time histogram. The end result is a model with several closed states and several open states in the form of a molecular kinetic scheme.

Figure 2.2: Single-channel patch clamp recording of a big potassium (BK) channel activated by calcium. Upward inflections represent an opening of the channel. From this type of trace, open-time and closed-time distributions can be obtained. Adapted from (12).

Figure 2.3: Examples of molecular kinetic models describing (A) the phosphorylation and dimerization of the signaling molecule signal transducer and activator of transcription 1 (STAT1), and (B) single-cell epigenomic state changes that lead to the transcription of a particular gene.

These Markov-based molecular kinetic schemes, although initially used to describe ion channels, constitute a simple and flexible model to describe biological processes in which different molecules change states, or single cells transitioning between different functional states (Fig. 2.3).

2.2 Classic Biophysical Problem

The problem of uniqueness of possible molecular kinetic models has been largely studied in the ion-channel field as well as others (13–18). It was soon established that two different processes may be indistinguishable from one another if only a restricted amount of information is available about them, and as a result different models of ion-channel kinetics may produce identical observable outcomes, in particular at the macroscopic trace level (Fig. 2.4). As explained above, ion channel models commonly consist of a number of states that are classified as open or closed, with a certain probability. It can be shown that one can redefine the states in the channel model to form a new

Figure 2.4: Example of classic voltage-clamp experiment traces (A) and an example of two molecular kinetic schemes describing ion channel dynamics in response to a voltage step that yield similar macroscopic traces. C indicates the closed-channel state, O the open-channel state, I the inactivated-channel state, CI the closed-inactivated channel state, and OI the open-inactivated channel state.

model with the same observable statistics as the original model. Similarly and because a Markov model follows a linear differential equation, there is a class of similarity transformations that do not alter the observable statistics of the model (19–23). Models with the same observable properties are said to be 'equivalent models' (see (24) for a mathematical description of the similarity transformations), and although certain equivalent models only amount to a more detailed description with a series of lumped states, in some instances equivalent models are describe and are associated with functionally and intrinsically distinct biological properties.

2.3 Current Methods

Current methods to derive information from macroscopic traces fall into two broad categories: i) *general network methods* that generate networks, but not molecular kinetic schemes, from biological data, and ii) *underlying model methods* that can derive kinetic schemes from macroscopic traces, but require a detailed model description of the current of the ion channel under study.

2.3.1 General network methods

In the broader context of systems biology, other powerful methods exist to extract network models from biological data in different domains. As one could

expect, each method and approach has its strengths and is most adapted to deal with specific aspects or features of the data and the biological system. Representative examples of these general network methods can be found in:

- **Boolean regulatory networks.** They have been particularly utilized to study immune system regulation. These methods extract a dynamical system model with parameters that can be estimated from genomic microarray or RNA-seq data (25, 26). They have been shown to obtain information regarding immune system gene regulatory networks (27, 28), albeit without detailing microscopic molecular processes.
- **Non-parametric Markov models.** These methods, exemplified by work from T. Thorne and M.P. Stumpf (29, 30), infer regulatory network structures that may vary between time points, using a set of hidden states that describe the network structure at a given time point. To model the distribution of the hidden states, these methods fit a Hierarchical Dirichlet Process Hidden Markov Model, a non-parametric extension of the traditional Hidden Markov Model, which does not require us to fix the number of hidden states in advance. The output network models are hierarchical, a feature that albeit useful for characterizing the responses of large sparsely sampled data sets, cannot easily be interpreted in terms of microscopic processes, as exemplified by this application to the study of V(D)J recombination in B cells (31).
- **Topological sensitivity methods.** Based on sensitivity analysis these methods develop a framework to systematically evaluate potentially vast sets of candidate differential equation models. This topological sensitivity analysis allows them to evaluate quantitatively the dependence of model inferences and predictions on the assumed model structures (32–34).
- **Bayesian inference methods.** These methods present a general framework for network inference and dynamical prediction using time course data that is rooted in non-linear biochemical kinetics. This is achieved by considering a dynamical system based on a chemical reaction graph with associated kinetic parameters. Both the graph and kinetic parameters are treated as unknowns and the inference is carried out within a Bayesian framework, which allows the prediction of dynamical behavior even when the underlying reaction graph itself is unknown or uncertain (35–37).

2.3.2 Underlying model methods

Various methods have been developed to derive molecular kinetic schemes from macroscopic current traces in the ion channel biophysics field. The best results have been obtained utilizing covariance and maximum likelihood methods.

- **Covariance methods.** This approach, referred to as covariance fitting (CVF) (38, 39), couples Q-matrix calculations with a maximum likelihood algorithm to fit macroscopic data directly to explicit models of the current. Unlike conventional sum-of-squares minimization, CVF fits both the magnitude of the recorded current and the strength of the correlations between different time points. These methods have slowly become less common and been replaced in the ion channel field by maximum likelihood methods.
- **Maximum likelihood methods.** The principle of these methods (40–43) is to formulate the nonstationary macroscopic current I_t as a random variable with a Gaussian probability distribution, and to maximize the likelihood of the data with respect to the estimated parameters. For a given model, the mean μ_t and variance V_t of the macroscopic current I_t are functions of the rate constants, channel count, unitary conductance, and experimental protocol. These functions can be complicated, and involve calculation of the exponential of a rate matrix Q obtained for all the transitions between all the states. The method is *direct* because the *a priori* assumptions about the topology as well as other constraints are explicitly specified in the model which also includes two main assumptions: i) the ion channels are identical and independent, and ii) the experimental variables (such as ligand concentration, voltage, etc.) are not functions of channel activity.

These methods strongly rely on underlying models to characterize ion channel current flow which, following decades of biophysics research, are usually available (12). Unfortunately, these methods cannot be easily generalized to most biological systems, for which reliable models of the molecular microscopic processes are typically unavailable and large numbers of measurements are impractical. Furthermore, many of the available methods are not able to tackle stimuli (inputs) with different dynamics, such as exponential increases or decreases in the magnitude of the stimulus, a key feature of many biological systems and physiological responses.

2.3.3 SYSMOLE

As mentioned in the previous section, general network methods do not require any *a priori* information regarding the biological process, but their results are difficult to interpret in terms of microscopic biological process. In contrast, the underlying model methods provide results that are perfectly interpretable in terms of microscopic process, but suffer from having to have an *a priori* mathematical description of the molecular underpinnings of the process under study.

The goal of SYSMOLE, the tool presented in this thesis, is to provide a flexible method that capitalizes on and incorporates a certain level of biological *a priori* information while providing an interpretable model, albeit not detailed, of the biological processes of interest.

Summary and Conclusions

- The problem of uniqueness of molecular kinetic schemes has been identified and studied for many decades in the ion channel field
- Current methods to derive information from macroscopic traces either provide network models that are difficult to interpret (general network methods), or require an extremely detailed knowledge of the biological process under study (underlying model methods).
- The SYSMOLE tool aims to strike a balance between biological interpretability and the required amount of *a priori* information regarding the biological process underlying the macroscopic trace.

Chapter 3

System Identification Approach: Identifier-Classifier- Molecular Kinetic Converter

Introduction. In this chapter we will present an overview of the system-identification approach utilized by SYSMOLE to derive molecular kinetic schemes from macroscopic traces.

3.1 General Approach

In this study, we have developed a general method, SYSMOLE, to obtain molecular kinetic schemes from macroscopic traces. SYSMOLE is a hybrid computational-analytical method that, because it does not assume any pre-existing model of the system, can be applied to experimental traces recorded, in principle, in any biological system.

In practical terms, to successfully derive the molecular kinetic scheme underlying a macroscopic trace, a method must perform three distinct tasks: a) it must accurately model the dynamics of the trace, b) it must ensure that this model is unique, and c) it must convert this mathematical model into a biologically interpretable model. SYSMOLE includes a module to tackle each of these tasks (Fig. 3.1). The Identifier Module accurately captures the dynamics of the macroscopic trace and summarizes this information in the form of a transfer function. The Classifier Module derives a block diagram, a description of how different processes are combined, that is uniquely matched

Figure 3.1: Modular organization of SYSMOLE and workflow of the method.

to the transfer function. Finally, the Molecular Kinetic Converter (MKC) Module utilizes both the transfer function and the block diagram to derive a molecular kinetic scheme that can be interpreted biologically in terms of conformational changes of proteins or chemical reactions.

3.2 Implementation Overview

- The Identifier Module takes an input $u(t)$ stimulus and an output $y(t)$ response macroscopic trace pair and relies on System Identification techniques to obtain the transfer function $G(s)$, which is characterized by the time constants of its poles (τ_{poles}) and zeros (τ_{zeros}) and a gain.
- The Classifier Module finds the configuration or block diagram associated with the transfer function $G(s)$ by solving multiple optimization problems.
- The Molecular Kinetic Converter Module uses the block diagram to constrain the problem of analytically deriving a molecular kinetic scheme from the transfer function $G(s)$, which otherwise would have multiple solutions.

Summary and Conclusions

- SYSMOLE is a flexible hybrid computational-analytical tool to derive molecular kinetic schemes from biological macroscopic traces.
- SYSMOLE is designed in a modular fashion and incorporates three modules: an Identifier Module, a Classifier Module, and Molecular Kinetic Converter Module.

Chapter 4

Identifier Module

Introduction. In this chapter we will describe the mathematical underpinnings of the Identifier Module in SYSMOLE. This module is in charge of accurately capturing the dynamics of the biological system’s response and summarizing it in the form of a transfer function.

4.1 Description

SYSMOLE aims to understand the response of a biological system (such as a cell or a group of cells) to stimuli (such as the presence of a hormone, a drug, or electrical stimuli), by breaking down macroscopic traces in terms of simple processes (i.e. activation, or inactivation of ion channels or cell-signaling pathways). SYSMOLE relies on System Theory, which has developed robust process-oriented approaches using transformed domains, such as Fourier, Laplace or Z, as an alternative functional description that simplifies the process of analyzing the behavior of the system. In particular, the Identifier Module relies on the Laplace transformed domain. The Laplace transformation from the time domain t to the frequency domain s transforms differential equations into algebraic equations and convolution into multiplication. This allows for a description of the response of the system in the frequency domain as a product $Y(s) = G(s) \cdot U(s)$, where $Y(s)$ and $U(s)$ are the output and the input in the frequency domain respectively and $G(s)$ the transfer function that characterizes the system. We can thus represent the response of the biological system to a particular stimulus by a transfer function that contains, in principle, all the information required to derive the system’s response to any input signal as long as certain constraints are met (see *section 4.2*). It should be noted that despite this input-output characterization not being suited for systems with multiple outputs to one input

such as bistable systems, it is still extremely efficient to characterize a wide range of biological systems and stimuli, as will be demonstrated herein.

The Identifier Module embodies the system-identification component of our approach. It uses the relationship between a given input trace $u(t)$ and the resulting output trace $y(t)$ to characterize the dynamics of the system and to summarize them in the form of an overall transfer function $G(s)$.

4.2 Mathematical Framework

The Identifier Module considers systems to be causal, linear, and time-invariant (LTI). Causality implies that the output at a certain time depends only on the input up to that time. Linearity enforces the output response of the system to a linear combination of inputs to be equal to the linear combination of the output responses to the individual inputs. Time-invariance means that the output response to a given input does not depend on absolute time.

Although on a first approach many biological systems might not seem to meet these properties, these assumptions can be met experimentally by applying some simple constraints. To ensure linearity, one can expose the system to a range of inputs (e.g. concentrations or membrane depolarizations) known not to saturate the system. To ensure time-invariance, the researcher can wait for the system to be at steady state before exposing it to the input and measure its response only once if long-term desensitization or other long-term memory processes are known to play a significant role in the system. Given this LTI characterization, SYSMOLE is best adapted to study systems whose behavior can be described or approximated by ordinary differential equations (ODEs). It should be noted that although not all biological processes can be approximated by ODEs, a large number of them are based on chemical reactions which historically have been characterized using this mathematical formalism. For reference, the European Biological Model Repository (BioModels, <https://www.ebi.ac.uk/biomodels/>)(44) contains 787 ODE models out of the 937 currently deposited.

The following subsections detail the mathematical formalism underpinning SYSMOLE's ability to obtain a transfer function in the frequency domain $G(s)$ from the system's input $u(t)$ and output $y(t)$ experimental macroscopic traces.

Figure 4.1: Identifier Module in SYSMOLE's Flow Chart.

4.2.1 Linear Time-Invariant (LTI) Systems

System theory defines a *system* as an input-output relationship. Here we will consider a system with a scalar input signal $u(t)$ and a scalar output signal $y(t)$. It is well known (see (45) for a review on the topic) that a causal linear time-invariant (LTI) system can be described by its impulse response $g(\tau)$ as follows

$$y(t) = \int_{\tau=0}^{\infty} g(\tau) u(t - \tau) d\tau \quad (4.1)$$

Knowing $g(\tau)$ from $\tau = 0$ to ∞ and knowing $u(t)$, we can compute the corresponding output $y(t)$ for any input. The impulse response is thus a complete characterization of the system.

For most biological systems, and given the limitations of experimental data-acquisition equipment, the researcher has access to inputs and outputs observations in discrete time. We thus assume $y(t)$ to be observed at the sampling instants $t_k = kT$ with $k = 1, 2, \dots$, where T is the sampling interval. In those terms, we can rewrite equation (4.1) as

$$y(t) = \int_{\tau=0}^{\infty} g(\tau) u(kT - \tau) d\tau \quad (4.2)$$

Assuming that $u(t)$ does not change much during a sample interval we can express $y(t)$ as

$$y(t) = \sum_{k=1}^{\infty} g(k)u(t-k) \quad (4.3)$$

where we ease the notation and assume that T is one time unit and use t to enumerate the sampling intervals.

We will use a shorthand notation for (4.3). To that effect, we introduce the *forward-shift* operator q defined as

$$qu(t) = u(t+1) \quad (4.4)$$

and the *backward-shift* operator q^{-1} defined as

$$q^{-1}u(t) = u(t-1) \quad (4.5)$$

Using these operators we can obtain the output of the system as

$$y(t) = \sum_{k=1}^{\infty} g(k)u(t-k) = \sum_{k=1}^{\infty} g(k)(q^{-k}u(t)) = \left[\sum_{k=1}^{\infty} g(k)q^{-k} \right] u(t) = G(q)u(t) \quad (4.6)$$

such that

$$G(q) = \sum_{k=1}^{\infty} g(k)q^{-k} \quad (4.7)$$

It is important to note that this is in formal agreement with the equivalent Z-transform expression for the impulse response of a LTI system

$$G(z) = \sum_{k=1}^{\infty} g(k)z^{-k} \quad (4.8)$$

Since the Z transformed domain is the discrete version of the continuous Laplace transformed domain, $G(q)$ contains all the necessary information to build from sampled sequences the transfer function $G(s)$. In addition, through $G(q)$ we can obtain the output sequence without perturbations $y^*(t)$ for any sampled input signal through the product:

$$y^*(t) = G(q)u(t) \quad t = 1, 2, \dots, N \quad (4.9)$$

4.2.2 Disturbances

Provided that the system's impulse response is known, according to the relationship (4.2) the output can be exactly calculated from the input. In most cases this is unrealistic. There are always signals beyond our control that affect the system. In our linear framework we will assume that those effects can be lumped into an additive term $v(t)$ at the output

$$y(t) = \sum_{k=1}^{\infty} g(k)u(t-k) + v(t) \quad (4.10)$$

Inputs could also be corrupted but we will include their effects on the output in $v(t)$ without loss of generality.

Disturbances can come from measurement noise, and other sources of biological noise (46). The most characteristic feature of the disturbance is that its value is not known beforehand, and therefore we will employ a probabilistic framework to characterize it. Mathematically, we will express $v(t)$ as the output of a system characterized by the impulse response $h(k)$:

$$v(t) = \sum_{k=0}^{\infty} h(k)e(t-k) \quad (4.11)$$

where the input $e(t)$ is a *stochastic process*, i.e. a sequence of independent identically distributed random variables. This process will be characterized by a certain probability density function (PDF) $f_e(x)$, where x is every value that the variable can take in the state space. PDF is typically not specified but rather described in terms of its first and second moments.

$$Ee(t) = \int x f_e(x) dx = 0 \quad (4.12)$$

$$Ee^2(t) = \int x^2 f_e(x) dx = \lambda \quad (4.13)$$

In practical terms, what do we need to characterize the disturbance $v(t)$? Typically we will not know the value of the disturbance $v(t)$ but rather we will have some information about its properties in the frequency domain. In other words, we will know its spectrum $\phi_v(\omega)$, where ω is the frequency. The Identifier Module assumes that the $e(t)$ that generated $\phi_v(\omega)$ is a process with $Ee(t)$ and $Ee^2(t)$ that are independent of time t (i.e. stationary) with values 0 and λ respectively. Then we can mathematically convert (4.11) into

$$\phi_v(\omega) = \lambda | H(e^{j\omega}) |^2 \quad (4.14)$$

where $H(e^{j\omega})$ is the Discrete Fourier Transform (DFT) of $h(k)$ (see (45) for a detailed description of the properties of this transform).

The next logical question becomes: is it reasonable to characterize a disturbance $v(t)$ by equation (4.11)? Or in other words, does $h(k)$ exist for any $\phi_v(\omega)$? We will rely on the *spectral factorization theorem* as proof of the existence of $h(k)$. This theorem imposes some conditions on $\phi_v(\omega)$. The theorem states that if $\phi_v(\omega)(> 0)$ is a rational function of $\cos(\omega)$ or $e^{j\omega}$, then there exists a monic rational function of z , $H(z)$ such that $H(0) = 1$, with no poles and no zeros on the outside unit circle satisfying

$$\phi_v(\omega) = \lambda |H(e^{j\omega})|^2 \quad (4.15)$$

where $H(z)$ is the Z-transform of $h(k)$. Given that the DFT is a particularization of the Z-transform for $z = e^{j\omega}$, $H(e^{j\omega})$ will also exist and satisfy

$$\phi_v(\omega) = \lambda |H(e^{j\omega})|^2 \quad (4.16)$$

Since $H(z)$ is monic, $h(k)$ will also need to be monic, i.e. $h(0) = 1$. However, this implies no loss of generality since the variance of $e(t)$ can be adjusted.

In summary, the Identifier Module assumes $e(t)$ to be a gaussian white-noise with a variance λ and therefore the spectral density $\phi_v(\omega)$ will allow us to define $h(k)$ and characterize the disturbance. Although $e(t)$ being gaussian might seem a constraining assumption, it still allows us to characterize a wide range of disturbances $v(t)$ by using different $h(k)$ sequences. Furthermore, this has been a successful disturbance characterization for the particular biological systems studied in this thesis, which include gene induction in response to infection (*Chapter 7*), calcium ion-channels in neurons (*Chapter 9*), and G protein-coupled receptor signaling (*Chapter 10*). Although not explored within the scope of this thesis, one could forecast that traces obtained from certain neural systems in which external noise enhances the signal response will not be characterized appropriately and will require other frameworks (47).

Going back to the compact notation, we can expand equation (4.9) to include the disturbance

$$H(q) = \sum_{k=1}^{\infty} h(k)q^{-k} \Rightarrow v(t) = H(q)e(t) \quad (4.17)$$

$$v(t) = H(q)e(t) \quad (4.18)$$

$$y(t) = y^*(t) + v(t) \quad (4.19)$$

where $v(t)$ is the disturbance generated by $e(t)$ in the system as determined by spectral factorization, and $y(t)$ the total output of the system.

Our basic description for a LTI system with additive disturbance will thus be

$$y(t) = G(q)u(t) + H(q)e(t) \quad (4.20)$$

4.2.3 Prediction of the Disturbance

As mentioned in the previous subsection, in order to obtain an accurate model of our system we will need to be able to predict the values of the disturbance $v(t)$ at time $t = t_1$ based on our previous knowledge of $v(t)$ for $t < t_1$. In the Identifier Module we predict the future values of $v(t) = H(q)e(t)$ as follows. First, we will need to impose two properties to $H(q)$ consistent with the spectral factorization theorem

- a) $H(q)$ should be stable.

$$\sum_{k=0}^{\infty} |h(k)| < \infty \quad (4.21)$$

- b) $H(q)$ should be invertible (invertibility of the noise-model). This implies that if $v(t)$ is known for $t < t_1$, there is a filter $\tilde{H}(q)$ such that

$$e(t) = \tilde{H}(q)v(t) = \sum_{k=0}^{\infty} \tilde{h}(k)v(t-k) \quad (4.22)$$

It can then be demonstrated (see (48)) that $\tilde{H}(q) = H(q)^{-1} = \frac{1}{H(q)}$ if the equivalent function in the Z plane $\frac{1}{H(z)}$ is analytical in $|z| > 1$ and $H(q)$ is stable.

One-step-ahead Prediction of $v(t)$

Suppose that we have observed $v(s)$ for $s \leq t-1$. We want to predict $v(t)$ based on these observations. We have, since $h(k)$ is monic, that

$$v(t) = \sum_{k=0}^{\infty} h(k)e(t-k) = e(t) + \sum_{k=1}^{\infty} h(k)e(t-k) \quad (4.23)$$

Taking into account (4.22) we can write

$$e(t) = \sum_{k=0}^{\infty} \tilde{h}(k)v(t-k) \quad (4.24)$$

and if we know $v(s)$ for $s \leq t-1$, we will also know $e(s)$ for $s \leq t-1$.

Let us define $m(t) = v(t) - e(t)$. Following (4.23) we can write $m(t)$ as a function of the observed past values of $m(t)$ up until time $t = t-1$, which we will denote by $m(t-1)$.

$$m(t) = \sum_{k=1}^{\infty} h(k)e(t-k) \quad (4.25)$$

If $\hat{v}(t)$ is the predicted disturbance sequence at time t (*prediction of $v(t)$*), the mean-square error of the prediction error can be mathematically expressed as $E(v(t) - \hat{v}(t))^2$. We will be interested in finding the $\hat{v}(t)$ that minimizes this error. It can be demonstrated (see (48) *Chapter 3*) that

- 1)

$$\min_{\hat{v}(t)} E(v(t) - \hat{v}(t))^2 \Rightarrow \hat{v}(t) = \hat{v}(t/t-1) \quad (4.26)$$

- 2)

$$\hat{v}(t/t-1) = m(t-1) \quad (4.27)$$

where $\hat{v}(t/t-1)$ is the most probable value of $v(t)$ given $v(s)$ with known values from $-\infty$ to $s = t-1$. This is also called *conditional expectation* of $v(t)$ or *maximum a posteriori* (MAP) prediction.

Therefore,

$$\hat{v}(t/t-1) = m(t-1) = \sum_{k=1}^{\infty} h(k)v(t-k) \quad (4.28)$$

where the minimization is carried out over all the functions $\hat{v}(t)$ with known past values up to $t = t-1$.

Since we cannot normally measure or have access to the values of $e(t)$ we can rewrite the predictor $\hat{v}(t/t-1)$ in terms of $v(t)$ as

$$\begin{aligned} \hat{v}(t/t-1) &= \left[\sum_{k=1}^{\infty} h(k)q^{-k} \right] e(t) = [H(q) - 1]e(t) = \\ &= \frac{H(q) - 1}{H(q)} v(t) = [1 - H^{-1}(q)]v(t) = \sum_{k=1}^{\infty} -\tilde{h}(k)v(t-k) \end{aligned}$$

Applying $H(q)$ to both sides we obtain

$$H(q)\hat{v}(t/t-1) = [H(q) - 1]v(t) = \sum_{k=1}^{\infty} h(k)v(t-k) \quad (4.29)$$

This expression of the one-step-ahead predictor of $v(t)$ will be extremely useful for the Identifier Module to create a model of the system and to help us determine its parameters (see next section *Models*). The one-step ahead predictor as defined is flexible and can be applied to different $v(t) = H(q)e(t)$ relationships. The following example shows its application for the *moving average* $H(q)$ defined by the relationship:

$$v(t) = e(t) + ce(t-1) \Rightarrow H(q) = 1 + cq^{-1} \quad (4.30)$$

Applying equation for the one-step ahead predictor $H(q)\hat{v}(t/t-1) = [H(q) - 1]v(t)$ we obtain

$$\hat{v}(t/t-1) + c\hat{v}(t-1/t-2) = cv(t-1) \quad (4.31)$$

and solving for the predictor

$$\hat{v}(t/t-1) = c[v(t-1) - \hat{v}(t-1/t-2)] \quad (4.32)$$

This expression allows us to calculate, for a noise model defined by $H(q)$, the next value of the disturbance $v(t)$ given its previous values.

One-step-ahead Prediction of $y(t)$

Similarly to the expression obtained for $v(t)$, we can obtain the one-step-ahead predictor for $y(t)$, i.e. the best prediction of $y(s)$ given that $y(s)$ and $u(s)$ are known for $s \leq t-1$.

We will start from the general expression for a LTI system given by equation $y(t) = G(q)u(t) + H(q)e(t)$. At time $t = s$ we know that $v(s) = y(s) - G(q)u(s)$ and since $u(s)$ and $y(s)$ are known for $s \leq t-1$, so will be $v(s)$. The predicted value for $y(t) = G(q)u(t) + v(t)$ will be the conditional expectation of $y(t)$ given the information we have. Mathematically:

$$\begin{aligned} \hat{y}(t/t-1) &= G(q)u(t) + \hat{v}(t/t-1) = \\ &= G(q)u(t) + [1 - H^{-1}(q)]v(t) = \\ &= G(q)u(t) + [1 - H^{-1}(q)][y(t) - G(q)u(t)] \end{aligned}$$

Rearranging we obtain

$$\hat{y}(t/t-1) = H^{-1}(q)G(q)u(t) + [1 - H^{-1}(q)]y(t) \quad (4.33)$$

or

$$H(q)\hat{y}(t/t-1) = G(q)u(t) + [H(q) - 1]y(t) \quad (4.34)$$

It is important to note that the probabilistic part of equation (4.20) is entirely included in the disturbance term $v(t)$, and therefore the knowledge of the predictor for $v(t)$ given by (4.28) and the relationship between the output and the disturbance from (4.20) are sufficient to provide an expression for $\hat{y}(t/t-1)$. These results can be generalized for a k -step-ahead predictors and will be used in order to include the effects of disturbances and estimate the model from the experimental input-output pairs.

4.3 Models

4.3.1 Transfer Function Models

In general, a linear time-invariant (LTI) model is therefore specified by:

1. The impulse response sequence $g(t)$ from $t=1 \cdots \infty$.
2. The spectrum of the additive disturbance, $\Phi_v(\omega) = \lambda |H(e^{j\omega})|^2$
3. The probability density function (PDF) of the disturbance $e(t)$, $f_e(x)$

A complete model is thus given by

$$y(t) = G(q)u(t) + H(q)e(t), \quad f_e(x) \quad (4.35)$$

with

$$G(q) = \sum_{k=1}^{\infty} g(k)q^{-k}, \quad H(q) = 1 + \sum_{k=1}^{\infty} h(k)q^{-k} \quad (4.36)$$

This formulation does not characterize a specific model, but rather yields a family of models. Rational transfer functions and finite-dimensional state-space descriptions are typical examples of models that belong to this family. In order to have a concrete model, we only need to specify $G(q)$, $H(q)$, and $f_e(x)$.

It is possible to determine the coefficients that define the polynomials $H(q)$, $G(q)$ in the equation (4.35) from input-output pairs of the system.

Mathematically, we can rewrite (4.35) in terms of the parameters to be estimated (vector θ) and obtain the following description

$$y(t) = G(q, \theta)u(t) + H(q, \theta)e(t) \quad (4.37)$$

where $f_e(x, \theta)$ is the PDF of $e(t)$, being $e(t)$ white noise.

For LTI models, the parameters that define $G(q)$ and $H(q)$ are real numbers, and the parameter vector θ ranges over a subset of $\mathbb{R}^d$ where d is the dimension of θ .

We will call (4.37) a *probabilistic model*, since this formulation gives a complete characterization of the probabilistic properties of the system. It should be noted that the probabilistic model requires the knowledge of $f_e(x, \theta)$. Using the one-step-ahead predictor from equation (4.33), the model can also be expressed as:

$$\hat{y}(t/\theta) = H^{-1}(q, \theta)G(q, \theta)u(t) + [1 - H^{-1}(q, \theta)]y(t) \quad (4.38)$$

Evidently, different ways of describing (4.37) or parametrizations in terms of θ are possible. The most immediate way of parametrizing G and H is to represent them as rational functions and let the parameters be the numerator and denominator coefficients. This type of parametrization yields a *transfer function model*, which is the one used by the Identifier module.

4.3.2 ARX Model Structure

Probably the most simple input-output relationship is obtained by describing the relationship between the input and the output traces as a linear difference equation

$$y(t) + a_1y(t-1) + \dots + a_nay(t-na) = b_1u(t-1) + \dots + b_nbu(t-nb) + e(t) \quad (4.39)$$

Since the white-noise term $e(t)$ enters here as a direct error in the difference equation, the model (4.39) is often called *equation error model structure*. The adjustable parameters are in this case

$$\theta = [a_1, a_2, \dots, a_{na}, b_1, \dots, b_{nb}]^T \quad (4.40)$$

Using the q operator introduced before for our formulation

$$A(q) = 1 + a_1q^{-1} + \dots + a_naq^{-na} \quad (4.41)$$

$$B(q) = b_1q^{-1} + \dots + b_nbq^{-nb} \quad (4.42)$$

we observe that in $y(t) = G(q, \theta)u(t) + H(q, \theta)e(t)$

Figure 4.2: The ARX model structure.

$$G(q, \theta) = \frac{B(q)}{A(q)} \text{ and } H(q, \theta) = \frac{1}{A(q)}$$

This model (4.39) is an ARX model, where AR refers to the autoregressive part $A(q)y(t)$ and X to the extra input $B(q)u(t)$ (called exogenous variable in econometrics). The block diagram for an ARX model is depicted in Figure 4.2. In the special case where $n_a = 0$, we have a set of models particularly common in signal processing that are characterized by a finite impulse response (FIR).

What is the one-step-ahead predictor for the ARX model structure? Using

$$\hat{y}(t/\theta) = G(q, \theta)u(t) + [H(q, \theta) - 1]y(t)$$

we obtain

$$\hat{y}(t/\theta) = B(q)u(t) + [1 - A(q)]y(t) \quad (4.43)$$

in which we have changed the nomenclature from $\hat{y}(t/t - 1)$ to $\hat{y}(t/\theta)$ to emphasize the dependency of the predictor with the parameter vector. From here on, all predictors will be one-step-ahead unless otherwise indicated. Now, we introduce the vector

$$\phi(t) = [y(t - 1), \dots, y(t - na), u(t - 1) \dots u(t - nb)]^T$$

and (4.43) can be re-written as

$$\hat{y}(t/\theta) = \theta^T \phi(t) = \phi^T(t) \theta \quad (4.44)$$

This constitutes an extremely important property of the ARX model. In light of (4.37) we see that the model is equivalent to a linear regression, which will

significantly simplify the parameter estimation of the model. Therefore, if an ARX structure is suited to model the input-output relationship under study, it is to our advantage to explore this type of model first in order to simplify calculations. It will be shown in later chapters that ARX constitutes an appropriate model to characterize the biological systems we have studied given the obtained experimental traces.

4.3.3 Parameter Estimation Problem

Mathematically we can summarize the parameter estimation problem as follows. We are now in the situation in which we have selected a certain model structure M (ARX in our case) with particular models $M(\theta)$ parametrized using a parameter vector $\theta \in D_M \subset \mathbb{R}^d$ where D_M is the subset of real values allowed for the parameters. The set of models thus defined is

$$M^* = M(\theta \mid \theta \in D_M). \quad (4.45)$$

We should recall that each model represents a way of predicting future outputs. In this sense, as we showed in the previous section, the model has a predictor associated with it. It should be noted that, in general, the predictor can be a linear or a non-linear combination of the input and the output (for a comprehensive review on predictors see *System Identification: Theory for the User* by Ljung (48)).

We are also in the situation in which we have collected data from the biological system sampling in time (an input and an output) that could be generally described by the vector

$$Z^N = [y(1), u(1), y(2), u(2), \dots, y(N), u(N)]$$

The problem we have to solve consists in using the information contained in Z^N to select a proper value $\hat{\theta}_N$ of the parameter vector space and hence a proper member $M(\hat{\theta}_N)$ in the set M^* . Formally speaking, we have to determine a mapping from the data Z^N to the set D_M

$$Z^N \longrightarrow \hat{\theta}_N \in D_M$$

How do we do this? We will choose a model and estimate the parameters such that they minimize the prediction error of the model.

Minimizing Prediction Errors

We will define the prediction error sequence

$$\varepsilon(t, \theta_N) = y(t) - \hat{y}(t/\theta_N) \quad (4.46)$$

which is a vector in $\mathbb{R}^N$. To measure its "size" we could use any norm in $\mathbb{R}^N$, quadratic or non quadratic. We will consider the following way of evaluating "how large" the prediction-error sequence is. Let the prediction-error sequence be filtered through a stable linear filter $L(q)$ obtaining the filtered prediction-error sequence

$$\varepsilon_F(t, \theta) = L(q)\varepsilon(t, \theta) \quad 1 \leq t \leq N \quad (4.47)$$

We will then we use the following norm

$$V_N(\theta, Z^N) = \frac{1}{N} \sum_{t=1}^N l(\varepsilon_F(t, \theta)) \quad (4.48)$$

where $l(\cdot)$ is a scalar-valued, typically positive function. The function $V_N(\theta, Z^N)$ is, for a given input-output vector Z^N , a well-defined scalar-valued function of the model parameter θ . This function is defined in a clever way that allows it to be minimized with respect to the parameter vector. The estimate $\hat{\theta}_N$ is then defined by the following minimization problem

$$\hat{\theta}_N = \hat{\theta}(Z^N) = \arg \min_{\theta \in D_M} V_N(\theta, Z^N). \quad (4.49)$$

The methods that use this way of estimating θ are common and are called prediction-error identification methods (PEM) and were used to implement the Identifier Module. V_N is called the loss function. Specific implementations will have different $l(\cdot)$ and $L(\cdot)$.

Prefiltering: Choice of $L(\cdot)$

The effect of the filter $L(\cdot)$ is to allow extra freedom in dealing with non-momentary properties of the prediction errors. Clearly, if the predictor is linear and time-invariant, and $y(t)$ and $u(t)$ scalars, then the result of filtering $\varepsilon(t)$ is equivalent to first filtering the input-output data and then applying the predictors.

The effect of $L(\cdot)$ is best understood when interpreted in the frequency-domain. It is clear, however, that by the use of $L(\cdot)$, effects of high-frequency

disturbances not essential to the modeling problem, or slow drift and the like, can be removed. In that sense $L(\cdot)$ acts like a frequency weighting filter.

If a model $y(t) = G(q, \theta)u(t) + H(q, \theta)e(t)$ is used, the filtered error $\varepsilon_F(t, \theta)$ is given by

$$\varepsilon_F(t, \theta) = L(q)\varepsilon(t, \theta) = [L^{-1}(q)H(q, \theta)]^{-1}[y(t) - G(q, \theta)u(t)] \quad (4.50)$$

The effect of prefiltering is thus identical to changing the noise model in equation (4.37) from $H(q, \theta)$ to

$$\overline{H}_L(q, \theta) = L^{-1}H(q, \theta) \quad (4.51)$$

For the implementation of the Identifier Module, we decided to confine ourselves to $L(q) = 1$, since the option of prefiltering is taken care of by the freedom in selecting $H(q, \theta)$.

Norm: Choice of $l(\cdot)$

For the choice of $l(\cdot)$, a first candidate would be a quadratic norm

$$l(\varepsilon) = \frac{1}{2}\varepsilon^2 \quad (4.52)$$

and thus the standard choice that was also chosen to implement the Identifier Module. Time-varying norms and norms depending on the parametrization can be used if more robustness against bad data is needed. In summary, the *quadratic prediction-error criteria* for LTI models can be summarized as follows

$$V_N(\theta, Z^N) = \frac{1}{N} \sum_{t=1}^N \frac{1}{2}\varepsilon^2(t, \theta) \quad (4.53)$$

with

$$\varepsilon(t, \theta) = H^{-1}(q, \theta)[y(t) - G(q, \theta)u(t)] \quad (4.54)$$

and therefore the problem to solve is

$$\hat{\theta}_N = \hat{\theta}_N(Z^N) = \arg \min_{\theta \in D_M} \frac{1}{N} \sum_{t=1}^N \frac{1}{2}\varepsilon^2(t, \theta) \quad (4.55)$$

Parameter Estimation for ARX Models

We found in previous sections that the ARX method employs the predictor

$$\hat{y}(t/\theta) = \phi^T(t)\theta \quad (4.56)$$

Here ϕ is the regression vector, which for the ARX structure is

$$\phi(t) = [-y(t-1) \cdots -y(t-na)u(t-1) \cdots u(t-nb)]^T$$

The prediction error in this case becomes

$$\varepsilon(t, \theta) = y(t) - \phi^T(t)\theta \quad (4.57)$$

and the criterion resulting from $L(q) = 1$ and $l(\varepsilon) = \frac{1}{2}\varepsilon^2$ gives us a loss function

$$V_N(\theta, Z^N) = \frac{1}{N} \sum_{t=1}^N \frac{1}{2} [y(t) - \phi^T(t)\theta]^2 \quad (4.58)$$

which is in fact the *least-squares* criterion for the linear regression (4.56). As mentioned before, the unique feature of this criterion is that it is a quadratic function in θ and that it can be therefore minimized analytically as

$$\hat{\theta}_N = \hat{\theta}_N(Z^N) = \arg \min_{\theta \in D_M} V_N(\theta, Z^N) = \left[\frac{1}{N} \sum_{t=1}^N \phi(t)\phi^T(t) \right]^{-1} \frac{1}{N} \sum_{t=1}^N \phi(t)y(t) \quad (4.59)$$

or with other numerical matrix methods generally used to solve least-squares problems. This makes the ARX an attractive model from a computational stand-point.

4.4 Implementation of the Identifier

The Identifier Module was implemented in MATLAB (www.mathworks.com) using the System Identification Toolbox. Given an experimental input-output trace pair, the Identifier Module obtains a transfer-function model of the system as follows:

1. It creates an *iddata* object with the input $u(t)$ and output $y(t)$ sequences previously sampled. The *iddata* object structures are data types used in MATLAB to store input-output relationships, which can be passed as arguments to the system identification functions.
2. It generates a case matrix including all the possible combinations of values of $n_a = 1, 2, \dots, 10$ (number of poles) and $n_b = 1, 2, \dots, 10$ (number of zeros + 1) as defined on the ARX model (4.39). The delay n_k is not fixed since our focus is to obtain the poles and zeros of the system. The case matrix as it is defined, implies that only polynomials of up to a degree 10 are allowed for $B(q)$ and $A(q)$. For our experimental traces this was sufficient, but the possible degree can be increased if

the model is found to lack accuracy (i.e., high V_N is obtained for all the models). It should be noted that there is a correlation between the number of processes and the degree of these polynomials (*see Chapter 6. Molecular Kinetic Converter*).

3. For every n_a and n_b pair the module obtains the "best" parametrization vector θ defined as the one that minimizes the loss function

$$V_N(\theta, Z^N) = \frac{1}{N} \sum_{t=1}^N \frac{1}{2} [y(t) - \phi^T(t)\theta]^2 \quad (4.60)$$

The least-squares problem associated with each minimization (*see previous section*) is solved by QR factorization. In our hands the ARX model implemented as described, provided satisfactory results for the experimental traces studied with loss function values $< 10^{-7}$ for all cases.

4. Finally, it selects from the case matrix the simplest model such that $n_a > n_b$, which achieves the lowest V_N . In order to simplify the model, polynomial coefficients $< 10^{-10}$ are considered 0, poles with complex or negative time constants τ are discarded, and poles smaller (faster) than the sampling period of the signal removed (*see Chapters 9 and 10* for descriptions of how the sampling was performed). In all the experimental traces evaluated, applying these rules yielded a model with lower n_a and n_b , yet with almost identical V_N , indicating that discarding these components was not at the expense of the module's ability to capture the main features of the dynamics of the system.

Summary and Conclusions

- The Identifier Module relies on system identification theory to extract a transfer function $G(s)$ from input and output macroscopic traces.
- Many biological systems can be modeled as LTI systems by applying some simple experimental constraints including avoiding saturation or experimental protocols that include memory phases.
- Disturbances are modeled as filtered white gaussian noise (WGN) added at the output without loss of generality.
- ARX models are computationally-efficient and provide a flexible model that is suited for the biological traces explored in this thesis.

Chapter 5

Classifier Module

Introduction. In this chapter we will describe the mathematical underpinnings of the Classifier Module in SYSMOLE. This module obtains a block diagram from the transfer function $G(s)$. The block diagram describes the system's response in terms of connected first-order processes and incorporates *a priori* knowledge about the biological processes involved in the biological system's response.

5.1 Description

The Classifier Module is the module in which the experimentalist's knowledge of the biological system under study is included to constrain the problem. It is implemented as a series of comparisons aimed to obtain the distinguishing features of the transfer function obtained by the Identifier Module and translate them into the correct relationship between biological processes summarized in a block diagram (Fig. 5.1).

5.2 Mathematical Framework

In the Laplace frequency domain used by SYSMOLE, each process i is described by a first-order system (associated with a first-order ODE), with a transfer function $G_i(s)$ characterized by a gain parameter k_i and a time constant τ_i as follows:

$$G_i(s) = \frac{b_i}{s + \omega_i} \quad (5.1)$$

where $\omega_i = \frac{1}{\tau_i}$ and $b_i = \frac{k_i}{\tau_i}$. Given two processes a and b described by first-order systems, and thus by first-order transfer functions $G_a(s)$ and $G_b(s)$ (e.g., those depicted in Fig. 5.2) one can find only three truly different

Figure 5.1: Classifier Module in SYSMOLE's Flow Chart

ways to combine them: cascade, feedback, and parallel (Fig. 5.3).

We will refer to these configurations as canonical, since any combination of two first-order processes can be converted to one of these three configurations with a simple change in parameters (49, 50). Each canonical configuration has a corresponding block diagram as defined in Systems Theory (45) (Fig. 5.3, left) and yields a response with different dynamics (Fig. 5.3, right). Combining two first-order processes results in a second-order transfer function, which is characterized by two poles and one or no zeros. The dependence of the poles, zeros, and gain of $G(s)$ on the parameters τ_a , τ_b , k_a and k_b of the two first-order processes differs greatly between the three canonical configurations and provides each configuration with a characteristic signature (Fig. 5.4).

Given two first-order systems $G_a(s)$ and $G_b(s)$ described by first-order transfer functions $G_a(s) = \frac{b_a}{s + \omega_a}$ and $G_b(s) = \frac{b_b}{s + \omega_b}$, one can analytically derive the transfer functions resulting from combining these two processes following each canonical configuration and obtain:

$$G(s)_{\text{cascade}} = \frac{b_a b_b}{(s + \omega_a)(s + \omega_b)} \quad (5.2)$$

$$G(s)_{\text{feedback}} = \frac{b_a(s + \omega_b)}{(s + \omega_a)(s + \omega_b + b_b)} \quad (5.3)$$

Figure 5.2: Example of two first-order processes, a and b , described by first-order transfer functions $G_a(s)$ and $G_b(s)$ with parameters $\tau_a = 5$ ms, and $k_a = -1$, and $\tau_b = 100$ ms, and $k_b = 1$, and their responses in time (outputs $y_a(t)$ and $y_b(t)$) to an ideal step input at $t = 30$ ms ($u(t)$)

$$G(s)_{parallel} = \frac{(b_a + b_b)s + b_a\omega_b + b_b\omega_a}{(s + \omega_a)(s + \omega_b)} \quad (5.4)$$

As discussed above, each of these transfer functions has distinct features in terms of poles, zeros, and gain (τ_{pole1} , τ_{pole2} , τ_{zero} and $Gain$). The Classifier Module capitalizes on these differences and determines which canonical configuration corresponds to the transfer function obtained by the Identifier Module, $G(s)$, by comparing the transfer function features of $G(s)$ to those of the canonical $G(s)_{cascade}$, $G(s)_{feedback}$, and $G(s)_{parallel}$.

5.3 Implementation

The Classifier Module is implemented as a series of comparisons between the transfer function obtained from the Identifier Module and the canonical transfer functions (5.2), (5.3) and (5.4), as seen in Fig. 5.5. It tests sequentially whether the transfer function features for a particular configuration are found in $G(s)$. Transfer functions arising from systems with more than two processes are characterized by more than two poles. Similarly first-order transfer functions are characterized by having only one pole and no zeros and can be easily distinguished. From the second-order transfer functions, characterized by two poles, those arising from a cascade configuration have no zeros and are easily classified as well. However, discerning between parallel and feedback configurations constitutes a more challenging task.

Figure 5.3: Block diagram (left) and corresponding output $y(t)$ (right) in response to a step input $u(t)$ of the first-order processes $G_a(s)$ and $G_b(s)$ combined following the three canonical configurations: cascade, feedback and parallel.

Figure 5.4: Signature of poles, zeros, and gain. Second-order transfer-function values of the time constants associated with the poles and zero, and the gain resulting from applying the three canonical configurations to combine two first-order processes defined by time constants $\tau_a = 5$ ms and $\tau_b = 80$ ms and strengths (gains), k_a and k_b , ranging from -10 to 10.

5.3.1 Parallel vs. Feedback

It can be mathematically demonstrated that for every transfer function $G(s)$ with two poles and one zero arising from two first-order processes, one can find a set of first-order systems $G_{af}(s)$ and $G_{bf}(s)$ which, when combined in feedback, will result in $G(s)$ (see equation 5.7 below). It can also be demonstrated that one can find an analogous set of first-order systems $G_{ap}(s)$ and $G_{bp}(s)$ which, when combined in parallel, will result in $G(s)$. The Classifier Module distinguishes the parallel and feedback second-order configurations by solving two optimization problems with constraints on the parameters that characterize the first-order systems $G_a(s)$ and $G_b(s)$ that give rise to the second-order system. These parameters are b_a , b_b , ω_a and ω_b and the constraints defined as $\omega_{amin} \leq \omega_a \leq \omega_{amax}$, $b_{amin} \leq b_a \leq b_{amax}$, $\omega_{bmin} \leq \omega_b \leq \omega_{bmax}$, and $b_{bmin} \leq b_b \leq b_{bmax}$. The choice of these constraints is guided by the prior information available regarding the two biological processes of interest, e.g. knowing that one process is one order of magnitude faster than the second process. Prior information regarding the processes involved in the system is often known, however if no prior information is available the optimization problems will not be appropriately constrained (see *Chapter 11 section 11.2 Limitations*) More formally, one can derive a set of equations that define the region of the parameter space that should be avoided in order to ensure accurate classification as follows.

The transfer function resulting from the combination in parallel of two first-order systems $G_{ap} = \frac{b_{ap}}{s + \omega_{ap}}$ and $G_{bp} = \frac{b_{bp}}{s + \omega_{bp}}$ can be expressed as:

$$G_p(s) = \frac{(b_{ap} + b_{bp})s + b_{ap}\omega_{bp} + b_{bp}\omega_{ap}}{(s + \omega_{ap})(s + \omega_{bp})} \quad (5.5)$$

Analogously, the transfer function resulting from the combination in feedback of two first-order systems $G_{af} = \frac{b_{af}}{s + \omega_{af}}$ and $G_{bf} = \frac{b_{bf}}{s + \omega_{bf}}$ can be expressed as:

$$G_f(s) = \frac{b_{af}(s + \omega_{bf})}{(s + \omega_{af})(s + \omega_{bf} + b_{bf})} \quad (5.6)$$

By comparing each coefficient in the transfer function we can obtain the expressions of the set of parameters b_{ap} , b_{bp} , b_{af} , b_{bf} , ω_{ap} , ω_{bp} , ω_{af} , and ω_{bf} that would yield the same transfer function ($G_p(s) = G_f(s)$).

$$\begin{cases} \omega_{ap} = \omega_{af} \\ \omega_{bp} = \omega_{bf} + b_{bf} \\ b_{ap} = b_{af} - b_{bp} \\ b_{bp} = \frac{b_{af}\omega_{bf} + b_{ap}\omega_{bp}}{\omega_{ap}} \end{cases} \quad (5.7)$$

Consequently, the region of parameter space explored by the Classifier Module and defined by the constraints, should exclude values of b_{ap} , b_{bp} , b_{af} , b_{bf} , ω_{ap} , ω_{bp} , ω_{af} , and ω_{bf} that satisfy the above equations. In practice, one can find that this holds true when prior information about the time scales of the processes under study is included in the choice of the constraints. Typically we are able to provide a range for the speed and strength of each process under study and therefore adding this type of constraint does not constitute a complex task. As an example, from the trace depicted on the introductory figure (Fig. 2.4A), one can easily constrain the activation process (fast downward inflection) to have a time constant ranging from 1 to 50 ms, and the inactivation process (slow upward inflection) to have a time constant greater than 50 ms. Similarly, one can restrict the exploration of the parameter space to activation and inactivation gains between -0.2 to 0.2 nA/mV.

5.3.2 Optimization Problems

The Classifier Module then decides between the feedback and parallel configurations by solving two constrained optimization problems (Fig. 5.5). These two problems are defined by comparing the second-order transfer function $G(s)$ characterized by a 4-parameter set, either $(\tau_{pole1}, \tau_{pole2}, \tau_{zero}$ and $Gain)$ or $(B_1, B_0, A_1, \text{ and } A_0)$,

$$G(s) = \frac{B_1 s + B_0}{s^2 + A_1 s + A_0} \quad (5.8)$$

to $G(s)_{parallel}$ and $G(s)_{feedback}$.

We define the parallel optimization problem by comparing (5.8) to (5.5). Solving the problem thus amounts to determining whether there is a solution for the following system of equations within the range of values allowed for the parameters ω_a , ω_b , b_a , and b_b

$$\begin{cases} B_1 = b_a + b_b \\ B_0 = b_a \omega_b + b_b \omega_a \\ A_1 = \omega_a + \omega_b \\ A_0 = \omega_a \omega_b \end{cases} \quad (5.9)$$

Computationally we solve this problem as an optimization problem in which we minimize the quadratic cost function derived from the system of equations

Figure 5.5: Classifier Module implementation. Flow chart of the Classifier Module implementation of the comparison between $G(s)$, the transfer function obtained by the Identifier Module, and the transfer functions associated with the canonical configurations. This module tests sequentially whether $G(s)$ incorporates the features of a transfer function describing a higher-order system, a first-order system, or a second-order system in each of the canonical configurations: cascade, feedback or parallel. The cost function values for the parallel and feedback optimization problems, f_{valp} and f_{valf} respectively, are used to discern between these two canonical configurations (see subsection 5.3.1 *Parallel vs. Feedback*).

in (5.9)

$$f_{valp}(b_a, b_b, \omega_a, \omega_b) = \alpha_1 |B_1 - (b_a + b_b)|^2 + \alpha_2 |B_0 - (b_a \omega_b + b_b \omega_a)|^2 + \dots \\ \dots + \alpha_3 |A_1 - (\omega_a + \omega_b)|^2 + \alpha_4 |A_0 - \omega_a \omega_b|^2 \quad (5.10)$$

constrained to $\omega_{amin} \leq \omega_a \leq \omega_{amax}$, $b_{amin} \leq b_a \leq b_{amax}$, $\omega_{bmin} \leq \omega_b \leq \omega_{bmax}$, and $b_{bmin} \leq b_b \leq b_{bmax}$.

We define analogously the feedback optimization problem by comparing (5.8) to (5.6).

$$\begin{cases} B_1 = b_a \\ B_0 = b_b \omega_b \\ A_1 = \omega_a + \omega_b + b_b \\ A_0 = \omega_a (\omega_b + b_b) \end{cases} \quad (5.11)$$

Again, we can computationally find the solution as an optimization problem in which we minimize the following quadratic cost function derived from the system of equations in (5.11)

$$f_{valf}(b_a, b_b, \omega_a, \omega_b) = \alpha_1 |B_1 - b_a \omega_b|^2 + \alpha_2 |B_0 - b_a \omega_b|^2 + \dots \\ \dots + \alpha_3 |A_1 - (\omega_a + \omega_b + b_b)|^2 + \alpha_4 |A_0 - \omega_a (\omega_b + b_b)|^2 \quad (5.12)$$

constrained to $\omega_{amin} \leq \omega_a \leq \omega_{amax}$, $b_{amin} \leq b_a \leq b_{amax}$, $\omega_{bmin} \leq \omega_b \leq \omega_{bmax}$, and $b_{bmin} \leq b_b \leq b_{bmax}$.

The cost function values f_{valf} and f_{valp} provide a quantitative measure of the match between $G(s)$ and $G(s)_{feedback}$, and $G(s)_{parallel}$ respectively, provided that the constraints are sufficient to distinguish both processes (see above). A value of f_{valf} greater than f_{valp} indicates that $G(s)$ arose from two processes combined in parallel. Conversely, a value of f_{valp} greater than f_{valf} indicates a transfer function $G(s)$ associated with two processes combined following the feedback configuration (Fig. 5.5).

5.3.3 Simulations

The parallel and feedback optimization problems are implemented utilizing a trust-region-reflective algorithm (51) included in the Optimization Toolbox of Matlab. We studied the convergence properties of these two optimization problems for a large number of combinations and a wide value range for

Figure 5.6: Study of the convergence properties of the parallel and feedback optimization problems when tested with two processes, a and b , characterized by $\tau_a = 15$ ms and $\tau_b = 150$ ms combined in parallel (**A**) with $k_a \in [-10, 10]$ and $k_b \in [-10, 10]$, or in feedback (**B**) with $k_a \in [-10, 10]$, and $k_b \in [0, 10]$ (negative k_b yields unstable positive feedback systems). Left panel indicates in red the value for the cost function of the parallel problem (f_{valp}) and in blue the value for the cost function of the feedback problem (f_{valf}) as a function of the number of iterations in the optimization algorithm. First column indicates the normalized Euclidean distance between the real parameter values (τ_a, τ_b, k_a, k_b) to the solution obtained by the parallel optimization algorithm (top) and the feedback optimization problem (bottom). Second column indicates the values for the cost functions of the parallel optimization problem f_{valp} (top) and the feedback optimization problem f_{valf} (bottom). Finally, the third column indicates the value of the logical condition tested by the Classifier Module to discriminate between parallel and the feedback configurations (see Fig. 5.5). Identical analyses were performed testing the Classifier Module with all possible configurations for $\tau_a = [5, 10, 15, 20]$ and $\tau_b = [100, 150, 200, 300]$ with similar results. Boundary conditions used for optimization problems are k_a and $k_b \in [-20, 20]$, $\tau_a \in [1, 50]$ ms and $\tau_b \in [50, 350]$ ms. Note that we restricted $k_b \in [0, 20]$ for the feedback problem since negative k_b would yield positive-feedback unstable systems.

parameters τ_a , τ_b , and k_b generated by a Synthetic Trace Simulator. Two implementations were used for the Synthetic Trace Simulator, one in Matlab and one using COPASI (52), a software application for simulation and analysis of biochemical networks and their dynamics.

Our results confirmed the robustness of this implementation in the Classifier Module to discern between parallel and feedback configurations (Fig. 5.6). Similar principles can be applied to third-order systems by including more optimization problems in the implementation (see *Chapter 8* for a description of the implementation of the Classifier Module for transfer functions with three poles and two zeros).

Summary and Conclusions

- The Classifier Module in SYSMOLE capitalizes on the specific signature of poles, zeros, and gain associated with each canonical configuration: cascade, feedback and parallel.
- The Classifier Module is implemented as a series of comparisons between the transfer function obtained from the Identifier Module, and the transfer function associated with each canonical configuration.
- In order to distinguish between parallel and feedback configurations, optimization problems that incorporate *a priori* information regarding the biological processes under study need to be solved.
- Parallel and feedback optimization problems converge for a wide range of parameters and the implementation of the Classifier Module is capable of discerning between the parallel and feedback configurations for second-order transfer functions.

Chapter 6

Molecular Kinetic Converter

Introduction. This chapter provides a mathematical description of the analytical component of the SYSMOLE tool: the Molecular Kinetic Converter Module. This module derives a molecular kinetic scheme from the transfer function $G(s)$ obtained by the Identifier Module and the block diagram obtained by the Classifier Module (Fig. 6.1).

6.1 Description

In general, obtaining a molecular kinetic scheme associated with a given transfer function represents an underdetermined problem with multiple possible solutions (24). In this chapter we illustrate how we can use biological principles and our prior information about the biological system under study to constrain the problem and obtain a unique solution. This task, which is analytical as opposed to computational, is implemented by the Molecular Kinetic Converter Module (MKC).

Implementing the biological constraints in mathematical/analytical terms is not always straightforward. The MKC is implemented as a set of rules or steps that impose different biological/physical principles on the transfer function obtained by the Identifier Module, and are based on the block diagram (configuration) derived by the Classifier Module for that particular transfer function. It should be emphasized that the MKC strictly derives a Markov-chain state model and that the interpretation in molecular terms of each state will depend on the experimental conditions in which traces are obtained.

Figure 6.1: Molecular Kinetic Converter Module in SYSMOLE's Flow Chart.

6.2 Implementation Principles

Our methodology converts the block diagram (configuration) into a Markov-chain molecular kinetic scheme by implementing the following four principles:

1. *Number of States and Possible Transitions.* The number of processes that can be extracted from the input and output traces is established by the order of the transfer function. The relationship between these processes in the biological system, which is determined by the configuration, defines the possible transitions in the system.
2. *Mass conservation.* Mass should be conserved and thus the total amount of molecules, which is the sum of the occupancies of all the states in the system, has to remain constant. It is important to take into account that although ideally all possible states should be described, additional biologically possible states not detected by our experimental assay might be lumped into one state. Furthermore, if one of the processes is removing a given species from the system, for example through degradation, a *degraded* state will need to be included.
3. *Microscopic Reversibility.* This principle states that, corresponding to every individual set of transitions that takes a molecule through several states back to its original state (forward drive), there is a reverse set of transitions (reverse drive) such that in a state of equilibrium the average rate of the forward drive is equal to the average rate of its reverse drive.

In other words, although the macroscopic law of mass action reactions can be used to describe processes that perpetually circle through states, at the molecular level the microscopic reversibility principle states that molecules do not possess infinite energy to remain in constant motion. In our case, a constant circular flow of transitions involving three or more states would contradict this principle and thus would result in a molecular kinetic scheme not biologically possible (53–55).

4. *Observable State.* Our experimental assay will typically only allow us to measure one of the states of the biological system, such as the open state of a channel or the activated form of a protein. This observable state should be identified since its occupancy as a function of time is related directly to the measured value of the output signal.

6.3 Steps

An n -order transfer function $G(s)$ together with the block diagram that gives rise to the system it describes can be converted to a molecular kinetic scheme applying a set of steps and rules. As mentioned in previous chapters, in this context, block diagrams are defined following the classic engineering system theory. They will thus represent the interconnection of n first-order systems described by transfer functions characterized by a gain parameter (k_i) and a time constant (τ_i) as

$$G_i(s) = \frac{b_i}{s + \omega_i} \quad i = 1..n \quad (6.1)$$

where $b_i = \frac{k_i}{\omega_i}$ and $\tau_i = \frac{1}{\omega_i}$.

The connectivity constraint introduced by the block diagram allows our method to provide a one-to-one mapping between a given $G(s)$ and its associated molecular kinetic scheme. As such, the molecular kinetic scheme associated with the transfer function $G(s)$ which satisfies the previous four principles can be derived as follows:

1. Identify the nodes in the block diagram. The number of states (N_s) is equal to the number of nodes $= n + 1$. In the context of this analytical tool, nodes are the points in the block diagram located at the entrance and at the exit of each block (operation blocks like addition and subtraction only add one node). It should be noted that these rules provide the molecular kinetic scheme with the smallest number of states to represent the number of detected first-order processes n . More states might exist at the microscopic level that would be collapsed into

one of the states. The fractional occupancy in each state i is described by a time-dependent state variable $z_i(t)$ with $i = 1 \dots n + 1$.

2. Include transitions between nodes for each connection described in the block diagram with rates σ_k with $k = 1 \dots r$. The value of the signal at a node in a block diagram can be negative, however negative fractional occupancies of a state are not interpretable. In order to implement negative values and subtractions in the molecular kinetic scheme, we will need to invert the flow of the signal and the transition.
3. Check whether the principle of microscopic reversibility is satisfied by the molecular kinetic scheme. If any perpetual circular flows involving three or more states are present, offset it by adding an equivalent flow in the opposite direction using only the kinetic parameters of the system σ_k , to avoid increasing the number of degrees of freedom r .
4. Generate the following system of equations in the time domain:
 - $N_s - 1$ Ordinary Differential Equations (ODEs) describing the transitions between the states of the form

$$\frac{dz_i(t)}{dt} = \sum_{x=1}^l \sigma_x z_x(t) - \sum_{y=1}^m \sigma_y z_y(t) \quad i = 1 \dots N_s - 1 \quad (6.2)$$

where l is the number of transitions coming into the state i and m is the number of transitions leaving the state i . These equations follow the hypothesis of a Markov chain (i.e. the rates σ are independent of time and the state variables $z_i(t)$) and can be assimilated to Kirchhoff's First Law for circuit analysis.

- 1 equation relating the observable state variable $z_{obs}(t)$ to the output $y(t)$ through a proportionality constant γ (Observable Equation)

$$y(t) = \gamma z_{obs}(t) \quad (6.3)$$

Although for the experimental traces analyzed in the frame of this thesis (see *Chapters 9* and *10*) a simple proportionality constant was a sufficient model, it is not required for the output to be strictly proportional to the observation. If the relationship between the observed state and the measurement is known and can be characterized by another transfer function in the frequency domain $\gamma(s)$ it can then be explicitly included in the relationship (6.3).

- 1 equation that establishes mass conservation. (i.e. the changes in $z_i(t)$ are only due to the input signal). It is hard to implement the input signal in Markov chain models. Through the mass-conservation equation we can include the input $u(t)$ as the unique force driving the change in number of molecules in each state as a function of time $z_i(t)$ of the system that we assume to be initially at steady state. By enforcing

$$u(t) = \sum_{i=1}^{n+1} z_i(t) \quad (6.4)$$

we guarantee that the total number of molecules in the system is equal to the total amount of molecules before the input, together with the molecules provided by the input. This also ensures that molecules are neither created nor destroyed. For an input step, $u(t)$ is a constant that we can normalize to 1, and $z_i(t)$ can be interpreted as fractional occupancies in the classic ion channel sense (see *Chapter 2*). For simplicity, and given that our thesis focuses on experimental systems in which the input is a step, we will refer to $z_i(t)$ as fractional occupancies hereinafter. This can however be extended to inputs with any type of dynamics, which is one of the strengths of the method. It should be emphasized that the principles of mass conservation and microscopy reversibility are only imposed on the state fractional occupancies which are affected by a particular stimulus or input under study. Other states whose fractional occupancies remain constant or at steady-state could exist but are not included in the description since they neither affect the principles of mass conservation and microscopic reversibility, nor contribute to the response to the stimulus.

5. Laplace transform the system of equations

$$\begin{cases} sZ_i(s) - z_i(0) = \sum_{x=1}^l \sigma_x Z_x(s) - \sum_{y=1}^m \sigma_y Z_y(s) & i = 1..n \\ Y(s) = \gamma Z_{obs}(s) \\ U(s) = \sum_{i=1}^{n+1} Z_i(s) \end{cases} \quad (6.5)$$

where $z_i(0)$ is the value of the state variable at the initial time. Typically, one can choose the states where $z_i(0) = 0$ to be in the differential equations to simplify solving the system. The "input" state, in which

the system has initially a fractional occupancy of 1, is then included in the mass conservation equation. It should be noted however that the approach can be applied for any initial distribution of fractional occupancies amongst states.

6. To obtain the transfer function of the kinetic model $G(s)_{kin}$ we isolate

$$\frac{Y(s)}{U(s)} = G(s)_{kin} = \frac{C \prod_{j=1}^{n-1} (s + D_j)}{\prod_{j=1}^n (s + A_j)} \quad (6.6)$$

where C , D_j and A_j are constants.

7. Using block algebra we obtain the transfer function in terms of the first-order systems

$$G(s) = \frac{c \prod_{j=1}^{n-1} (s + d_j)}{\prod_{j=1}^n (s + a_j)} \quad (6.7)$$

where c , d_j and a_j are constants.

8. Solve the following system of equations

$$\begin{cases} D_j = d_j & j = 1..n-1 \\ A_j = a_j & j = 1..n \\ G(s)_{kin}]_{s \rightarrow 0} = G(s)]_{s \rightarrow 0} \end{cases} \quad (6.8)$$

where the third equation in the system equals the steady state gain of $G(s)$ and $G(s)_{kin}$, for the parameters of the molecular kinetic scheme as defined for each configuration (see Figures 6.2, 6.3, 6.4, 6.5, and 6.6)

$$\sigma_k = f(k_i, \omega_i) \quad (6.9)$$

$$\gamma = f(k_i, \omega_i) \quad (6.10)$$

It is straightforward to test that these rules yield the correct molecular kinetic scheme. Any mistake in the steps will result in a system of equations without solutions at step 8 when comparing the coefficients on $G(s)_{kin}$ and the $G(s)$ derived from the block algebra (see *section 6.8* below).

In the following subsections we present a comprehensive study of the application of these rules to first-order systems and the three possible canonical block-diagrams for second-order systems: cascade, feedback, and parallel.

Figure 6.2: First-order System.

6.4 First-Order System

1. Number of states= $n + 1=2$.
2. We identify the nodes and include the transitions in the diagram (see Figure 6.2).
3. We check that the principle of microscopic reversibility is satisfied.
4. We build the system of equations

$$\begin{cases} \frac{dz_2(t)}{dt} = \sigma_1 z_1(t) & \text{ODE Transition 1} \\ y(t) = \gamma z_2(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) & \text{Mass Equation} \end{cases} \quad (6.11)$$

5. We Laplace transform the system

$$\begin{cases} sZ_2(s) = \sigma_1 Z_1(s) & \text{T1} \\ Y(s) = \gamma Z_2(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) & \text{M} \end{cases} \quad (6.12)$$

6. We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, O and M.

$$G(s)_{kin} = \frac{Y(s)}{U(s)} = \frac{\sigma_1 \gamma}{s + \sigma_1} \quad (6.13)$$

7. From block diagram we obtain $G(s)$

$$G(s) = \frac{b_a}{s + \omega_a} \quad (6.14)$$

Figure 6.3: Two First-order Systems G_a and G_b connected in Cascade.

8. Through comparison of the terms in $G(s)_{kin}$ and $G(s)$ we obtain the parameters of the molecular kinetic scheme

$$\sigma_1 = \omega_a \quad (6.15)$$

$$G(s)_{kin}|_{s \rightarrow 0} = \gamma = G(s)|_{s \rightarrow 0} = \frac{b_a}{\omega_a} \implies \gamma = \frac{b_a}{\omega_a} \quad (6.16)$$

6.5 Second-Order System: Cascade

1. Number of states= $n + 1=3$.
2. We identify the nodes and include the transitions in the diagram (see Figure 6.3).
3. We check that the principle of microscopic reversibility is satisfied.
4. We build the system of equations

$$\left\{ \begin{array}{ll} \frac{dz_3(t)}{dt} = \sigma_2 z_2(t) & \text{ODE Transition 1} \\ \frac{dz_2(t)}{dt} = \sigma_1 z_1(t) - \sigma_2 z_2(t) & \text{ODE Transition 2} \\ y(t) = \gamma z_3(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) + z_3(t) & \text{Mass Equation} \end{array} \right. \quad (6.17)$$

5. We Laplace transform the system assuming $z_2(0) = 0$ and $z_3(0) = 0$ for flexibility

$$\left\{ \begin{array}{ll} sZ_3(s) = \sigma_2 Z_2(s) & \text{T1} \\ sZ_2(s) = \sigma_1 Z_1(s) - \sigma_2 Z_2(s) & \text{T2} \\ Y(s) = \gamma Z_3(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) + Z_3(s) & \text{M} \end{array} \right. \quad (6.18)$$

Figure 6.4: Two First-order Systems G_a and G_b connected in Feedback.

6. We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, T2, O, and M.

$$G(s)_{kin} = \frac{Y(s)}{U(s)} = \frac{\gamma \sigma_1 \sigma_2}{(s + \sigma_1)(s + \sigma_2)} \quad (6.19)$$

7. From the block diagram we obtain $G(s)$

$$G(s) = \frac{b_a b_b}{(s + \omega_a)(s + \omega_b)} \quad (6.20)$$

8. We compare terms in $G(s)_{kin}$ and $G(s)$ and obtain the following equations

$$\sigma_1 = \omega_a \quad (6.21)$$

$$\sigma_2 = \omega_b \quad (6.22)$$

$$G(s)_{kin}|_{s \rightarrow 0} = k_a k_b = G(s)|_{s \rightarrow 0} = \gamma \implies \gamma = k_a k_b \quad (6.23)$$

6.6 Second-Order System: Feedback

1. Number of states= $n + 1=3$.
2. We identify the nodes and include the transitions in the diagram (see Figure 6.4). It should be noted that due to the subtraction operation

present in the block diagram, the signal flows in the opposite direction through G_a and G_b and this is reflected accordingly in the transitions depicted in the diagram.

3. We check that the principle of microscopic reversibility is satisfied.
4. We build the system of equations

$$\left\{ \begin{array}{ll} \frac{dz_3(t)}{dt} = \sigma_2 z_2(t) - \sigma_3 z_3(t) & \text{ODE Transition 1} \\ \frac{dz_2(t)}{dt} = \sigma_1 z_1(t) - \sigma_2 z_2(t) + \sigma_3 z_3(t) & \text{ODE Transition 2} \\ y(t) = \gamma z_2(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) + z_3(t) & \text{Mass Equation} \end{array} \right. \quad (6.24)$$

5. We Laplace transform the system assuming $z_2(0) = 0$ and $z_3(0) = 0$ for flexibility

$$\left\{ \begin{array}{ll} sZ_3(s) = \sigma_2 Z_2(s) - \sigma_3 Z_3(s) & \text{T1} \\ sZ_2(s) = \sigma_1 Z_1(s) - \sigma_2 Z_2(s) + \sigma_3 Z_3(s) & \text{T2} \\ Y(s) = \gamma Z_2(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) + Z_3(s) & \text{M} \end{array} \right. \quad (6.25)$$

6. We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, T2, O, and M

$$G(s)_{kin} = \frac{Y(s)}{U(s)} = \frac{\gamma \sigma_1 (s + \sigma_3)}{(s + \sigma_1)(s + \sigma_3 + \sigma_2)} \quad (6.26)$$

7. From the block diagram we obtain $G(s)$

$$G(s) = \frac{b_a (s + \omega_b)}{(s + \omega_a)(s + \omega_b + b_b)} \quad (6.27)$$

8. Comparing terms in $G(s)_{kin}$ and $G(s)$ we obtain the following equations

$$\sigma_1 = \omega_a \quad (6.28)$$

$$\sigma_2 = b_b \quad (6.29)$$

$$\sigma_3 = \omega_b \quad (6.30)$$

$$G(s)_{kin}|_{s \rightarrow 0} = \frac{\gamma \sigma_1 \sigma_3}{\sigma_1 (\sigma_3 + \sigma_2)} = G(s)|_{s \rightarrow 0} = \frac{\gamma \omega_a \omega_b}{\omega_a (\omega_b + b_b)} \implies \gamma = k_a \quad (6.31)$$

Figure 6.5: Two First-order Systems G_a and G_b added in Parallel.

6.7 Second-Order System: Parallel Addition

1. Number of states= $n + 1=3$.
2. We identify the nodes and include the transitions in the diagram (see Figure 6.5).
3. We check that the principle of microscopic reversibility is satisfied.
4. We build the system of equations

$$\left\{ \begin{array}{ll} \frac{dz_3(t)}{dt} = \sigma_2 z_1(t) - \sigma_3 z_3(t) & \text{ODE Transition 1} \\ \frac{dz_2(t)}{dt} = \sigma_1 z_1(t) + \sigma_3 z_3(t) & \text{ODE Transition 2} \\ y(t) = \gamma z_2(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) + z_3(t) & \text{Mass Equation} \end{array} \right. \quad (6.32)$$

5. We Laplace transform the system assuming $z_2(0) = 0$ and $z_3(0) = 0$ for flexibility

$$\left\{ \begin{array}{ll} sZ_3(s) = \sigma_2 Z_1(s) - \sigma_3 Z_3(s) & \text{T1} \\ sZ_2(s) = \sigma_1 Z_1(s) + \sigma_3 Z_3(s) & \text{T2} \\ Y(s) = \gamma Z_2(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) + Z_3(s) & \text{M} \end{array} \right. \quad (6.33)$$

6. We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, T2, O, and M

$$G(s)_{kin} = \frac{Y(s)}{U(s)} = \frac{\gamma \left(s + \frac{\sigma_1 \sigma_3 + \sigma_3 \sigma_2}{\sigma_1} \right)}{(s + \sigma_1)(s + \sigma_2 + \sigma_3)} \quad (6.34)$$

7. From the block diagram we obtain $G(s)$

$$G(s) = \frac{(b_a + b_b)s + b_a \omega_b + b_b \omega_a}{(s + \omega_a)(s + \omega_b)} \quad (6.35)$$

8. Comparing terms in $G(s)_{kin}$ and $G(s)$ we obtain the following equations

$$\sigma_1 = \frac{b_a + b_b}{k_a + k_b} \quad (6.36)$$

$$\sigma_2 = \omega_b - \sigma_1 \quad (6.37)$$

$$\sigma_3 = \omega_a \quad (6.38)$$

$$G(s)_{kin}|_{s \rightarrow 0} = \gamma = G(s)|_{s \rightarrow 0} = k_a + k_b \implies \gamma = k_a \quad (6.39)$$

6.8 Second-Order System: Parallel Subtraction

1. Number of states= $n + 1=3$.
2. We identify the nodes and include the transitions in the diagram (see Figure 6.6). Due to the subtraction operation in the block diagram, the signal flows in the opposite direction through G_b and the transitions between states reflect this accordingly (see Figure 6.6, left).
3. We observe that the resulting molecular kinetic scheme does not satisfy the principle of microscopic reversibility. We adjust the molecular kinetic scheme by adding a flow in the clockwise direction equal to the flow on the counterclockwise direction, and no additional independent kinetic parameters σ_i are added (see Figure 6.6, center).

Figure 6.6: Two First-order Systems G_a and G_b subtracted in Parallel. **(Left)** Initial molecular kinetic scheme obtained which does not satisfy the condition of microscopic reversibility. **(Center)** Molecular kinetic scheme where microscopic reversibility has been enforced but lacks a solution for σ_i and γ . **(Right)** Molecular kinetic scheme with microscopic reversibility and an additional state with solution for the kinetic parameters σ_i and γ .

4. We build the system of equations

$$\left\{ \begin{array}{ll} \frac{dz_2(t)}{dt} = \sigma_1 z_1(t) + \sigma_3 z_3(t) - 2\sigma_2 z_2(t) & \text{ODE Transition 1} \\ \frac{dz_3(t)}{dt} = \sigma_1 z_1(t) + \sigma_2 z_2(t) - 2\sigma_3 z_3(t) & \text{ODE Transition 2} \\ y(t) = \gamma z_2(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) + z_3(t) & \text{Mass Equation} \end{array} \right. \quad (6.40)$$

5. We Laplace transform the system assuming $z_2(0) = 0$ and $z_3(0) = 0$ for flexibility

$$\left\{ \begin{array}{ll} sZ_2(s) = \sigma_2 Z_1(s) + \sigma_3 Z_3(s) - 2\sigma_2 Z_2(s) & \text{T1} \\ sZ_3(s) = \sigma_1 Z_1(s) + \sigma_2 Z_2(s) - 2\sigma_3 Z_3(s) & \text{T2} \\ Y(s) = \gamma Z_2(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) + Z_3(s) & \text{M} \end{array} \right. \quad (6.41)$$

6. We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, T2, O, and M

$$G(s)_{kin} = \frac{Y(s)}{U(s)} = \frac{\gamma(s + 3\sigma_1)}{s^2 + 2s(\sigma_1 + \sigma_2 + \sigma_3) + 3(\sigma_2\sigma_3 + \sigma_1\sigma_3 + \sigma_1\sigma_2)} \quad (6.42)$$

7. From the block diagram we obtain $G(s)$

$$G(s) = \frac{(b_a + b_b)s + b_a\omega_b + b_b\omega_a}{(s + \omega_a)(s + \omega_b)} \quad (6.43)$$

8. Comparing terms in $G(s)_{kin}$ and $G(s)$ one can conclude that there is no set of kinetic parameters σ_i and γ such that $G(s)_{kin}$ and $G(s)$ are equal. We therefore add an additional non-observable state (see Figure 6.6, right) and build the new system of equations

$$\left\{ \begin{array}{ll} \frac{dz_2(t)}{dt} = \sigma_1 z_1(t) + \sigma_3 z_4(t) - (\sigma_3 + \sigma_2)z_2(t) & \text{ODE Transition 1} \\ \frac{dz_3(t)}{dt} = \sigma_2 z_1(t) + \sigma_3 z_4(t) - (\sigma_3 + \sigma_1)z_3(t) & \text{ODE Transition 2} \\ \frac{dz_4(t)}{dt} = \sigma_1 z_3(t) + \sigma_2 z_2(t) - 2\sigma_3 z_4(t) & \text{ODE Transition 2} \\ y(t) = \gamma z_2(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) + z_3(t) & \text{Mass Equation} \end{array} \right. \quad (6.44)$$

9. We Laplace transform the system assuming $z_2(0) = 0$ and $z_3(0) = 0$ for flexibility

$$\begin{cases} sZ_2(s) = \sigma_1 Z_1(s) + \sigma_3 Z_4(s) - (\sigma_3 + \sigma_2)Z_2(s) & \text{T1} \\ sZ_3(s) = \sigma_2 Z_1(s) + \sigma_3 Z_4(s) - (\sigma_3 + \sigma_1)Z_3(s) & \text{T2} \\ sZ_4(s) = \sigma_1 Z_3(s) + \sigma_2 Z_2(s) - 2\sigma_3 Z_4(s) & \text{T3} \\ Y(s) = \gamma Z_2(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) + Z_3(s) & \text{M} \end{cases} \quad (6.45)$$

10. We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, T2, T3, O, and M and simplifying the expression

$$G(s)_{kin} = \frac{Y(s)}{U(s)} = \frac{\gamma(s + \sigma_3)}{(s + \sigma_1)(s + \sigma_2 + \sigma_3)} \quad (6.46)$$

11. From the block diagram we had $G(s)$

$$G(s) = \frac{(b_a + b_b)s + b_a\omega_b + b_b\omega_a}{(s + \omega_a)(s + \omega_b)} \quad (6.47)$$

12. Comparing terms in $G(s)_{kin}$ and $G(s)$ we obtain the following equations

$$\sigma_1 = \omega_a \quad (6.48)$$

$$\sigma_2 = \omega_b - \sigma_3 \quad (6.49)$$

$$\sigma_3 = \frac{b_a\omega_b + b_b\omega_a}{b_a + b_b} \quad (6.50)$$

$$G(s)_{kin}|_{s \rightarrow 0} = \frac{\gamma\sigma_3}{\sigma_1(\sigma_2 + \sigma_3)} = G(s)|_{s \rightarrow 0} = k_a + k_b \implies \gamma = \frac{(k_a + k_b)\omega_a\omega_b}{\sigma_3} \quad (6.51)$$

Summary and Conclusions

- The MKC Module takes the transfer function $G(s)$ obtained by the Identifier Module and the block diagram determined by the Classifier Module and derives the appropriate molecular kinetic scheme defined by transitions between states and parameters γ and σ_i .
- The MKC Module is implemented as a series of steps and analytical transformations that incorporate biophysical principles (mass conservation, microscopic reversibility) in order to ensure a molecular kinetic scheme interpretable in biological terms.

Chapter 7

Robustness to Noise

Introduction. This chapter will focus on the robustness to different types of noise of the SYSMOLE tool and signal processing methods to improve it.

7.1 Different molecular kinetic schemes from similar macroscopic traces

We decided to test first the ability of SYSMOLE to tackle a general class of problems in biology: distinguishing whether two similar macroscopic traces arise from different molecular kinetic schemes (see *Chapter 2*). Elucidating the molecular mechanisms of ion-channel inactivation from macroscopic current traces constitutes a classic biophysics problem in this class (see (12, 24, 56) for a review on the topic). Many of these molecular mechanisms were identified in the eighties and the nineties for different types of ion channels through single-channel electrophysiology recordings, fluctuation analysis, and analysis of gating currents (6, 7, 53, 57, 58). However, analogous problems emerge in a great number of biological systems for which it is hard to design experiments to tease molecular schemes apart, or single-molecule experimental techniques are simply not yet available.

To test the robustness to noise, we used the Synthetic Trace Simulator, previously described in *Chapter 5*, *section 5.3.3*. This module generates a trace simulating each of the five molecular kinetic schemes associated with each configuration (Fig. 7.1): first-order, second-order cascade, second-order feedback, second-order parallel addition, and second-order parallel subtraction. For practical purposes, in our implementation each molecular kinetic scheme is characterized by the parameters of two first-order processes a and

Figure 7.1: Molecular kinetic schemes derived from block diagrams for first-order systems (**A**), second-order systems in cascade (**B**), second-order systems in feedback (**C**), second-order in parallel addition (**D**), and second-order systems in parallel subtraction (**E**) configurations. Red circles indicate the observable state for each configuration. Black and blue circles depict non-observable states.

b (k_a , k_b , τ_a , and τ_b) and the kinetic parameters σ_k and γ derived from the equations detailed in the previous chapter (*Chapter 6 Molecular Kinetic Converter*).

As a proof of concept, we used the Synthetic Trace Simulator to simulate voltage-clamp experiments and generate, in response to a step in voltage, the macroscopic current trace for molecular kinetic schemes that include first-order processes describing the activation and inactivation of ion channels (Fig. 7.2). Emulating the classic biophysics problem, we compared two extremely similar macroscopic traces arising from different molecular kinetic schemes (compare left panels on sections A and B in Fig. 7.2). The first simulated current trace (Fig. 7.2A) emerged from a molecular scheme in which inactivation affected both the closed and the open states of the channel (Fig 7.1D, black: closed state, blue: inactivated states, red: open state). The trace was generated with activation (a) and inactivation (b) processes defined by parameters $k_a = -5$, $k_b = 2$, $\tau_a = 5$ ms, and $\tau_b = 200$ ms. The second simulated current trace was generated with a molecular kinetic scheme in which inactivation only acted on the open state (Fig. 7.1C, black: closed state, red: open state, blue: inactivated state). This trace was obtained with a molecular kinetic scheme associated with first-order activation (a) and inactivation (b) processes with parameters $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms, and $\tau_b = 100$ ms. We then analyzed both traces with SYSMOLE: first we identified the dynamics of the macroscopic trace elicited by the voltage step and summarized them in a transfer function, then we classified to obtain the underlying configuration, and finally we obtained the corresponding molecular kinetic scheme using the MKC Module (Fig. 7.2).

Our results indicate that our methodology can accurately retrieve the molecular kinetic scheme of these two similar traces. In order to control for a possible bias associated with using the same environments for simulation and analysis, two different implementations of the Synthetic Trace Simulator were tested: one with Matlab, the environment used for SYSMOLE implementation, and COPASI (52), a software application for simulation and analysis of biochemical networks and their dynamics. Practically identical results were obtained with both implementations.

Figure 7.2: Similar traces from different molecular kinetic schemes. Application of SYSMOLE to two similar synthetic traces in response to an ideal step at $t = 30$ ms. The top trace (**A**) was generated with the Synthetic Trace Simulator by combining now in parallel two processes with parameters $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms and $\tau_b = 100$ ms. The correct molecular kinetic scheme is derived and the kinetic parameters obtained. The bottom trace (**B**) was also generated with the Synthetic Trace Simulator by combining in feedback two processes with parameters $k_a = -5$, $k_b = 2$, $\tau_a = 5$ ms and $\tau_b = 200$ ms. The correct molecular kinetic scheme was derived and the kinetic parameters obtained. Boundary conditions used in the Classifier Module are $\tau_a \in [1, 9]$ ms, $\tau_b \in [50, 250]$ ms $k_a \in [-20, 20]$, and $k_b \in [-20, 20]$ for the parallel problem and $k_b \in [0, 20]$ for the feedback problem since combinations in feedback with $k_b < 0$ are unstable.

7.2 Robustness of SYSMOLE to noise in the macroscopic trace

The previous section had concluded that the system-identification approach subject of this thesis (SYSMOLE) could be successfully applied to accurately elucidate the molecular kinetic scheme underlying a given macroscopic trace. However, these studies had been performed in the absence of noise, an unrealistic situation in any biological experimental setting. We decided to test the effects of noise on the performance of SYSMOLE.

The robustness to noise of SYSMOLE will strongly depend on the features of the signals of interest and the type of noise existing in the trace. A good approach to establish the reliability of SYSMOLE in the presence of noise for the types of experimental traces we are studying is to simulate them with the aid of the Synthetic Trace Simulator (see above) and test SYSMOLE when different levels and types of noise are added. Here we studied the effects of noise on the kinetic parameter values of the molecular kinetic scheme and the probability of error in classification of traces in which we observe a fast-activating process followed by a slower inactivation at the macroscopic level (Fig. 7.3). These traces are representative of the types of traces obtained in voltage-clamp experiments with L-type calcium channels, and in the GPCR-heteromer signaling measurements obtained and analyzed in subsequent chapters (*Chapters 9 and 10*). By inspection of the traces (Figs. 5.3 and 7.3) one can easily conclude that the two processes are not combined in cascade, since this would be incompatible with a signal that reverses direction in the y axis. Therefore, we focused our analysis on how noise affects the distinction between the feedback and parallel configurations, which is more prone to error due to the presence of noise in the trace. Noise tests were performed for all configurations and a wide range of combinations of first order parameters for the activation and inactivation processes.

Specifically, we generated synthetic traces involving two first-order processes characterized by parameters $\tau_a = 5$ ms, $\tau_b = 100$ ms, $k_a = 5$, and $k_b = 3$, using the molecular kinetic scheme associated with either the parallel subtraction (Fig. 7.1E) or the feedback (Fig. 7.1C) configurations. We generated multiple traces for each molecular kinetic scheme, to which we added increasing levels of white Gaussian noise ($N = 100$ simulations per level of noise). We analyzed each trace with SYSMOLE and measured (i) the relative error of the transition rate parameter values from the molecular kinetic schemes σ_1 , σ_2 and σ_3 (Figs. 7.1D and 7.1E), and (ii) the probability of error

Figure 7.3: Representative traces of output signal $y(t)$ used to test the effects of noise. Traces were generated with the Synthetic Trace Module combining two first-order processes with parameters $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms and $\tau_b = 100$ ms in parallel with different levels of white Gaussian noise added.

in classification (P_e) defined as the ratio between the number of correct classifications and the total number of simulations at a given level of noise (Fig. 7.4). It should be noted that the scaling factor is an adjustable parameter that shapes the amplitude of the signal and its sensitivity to noise has less relevance than the transition rates or the configuration.

7.2.1 Signal-to-noise ratio calculation

In order to obtain the signal-to-noise ratio (SNR) we need to know the average power for signal $y(t)$, P_Y , as well as the noise power, P_N . The signal's average power can be directly calculated in the time domain as:

$$P_Y = \frac{1}{N} \sum_{n=0}^{\infty} y[n]^2 \quad (7.1)$$

where $y[n]$ is the sequence resulting from discretizing $y(t)$ with sample period T_s and sample number $n = 1 \cdots N$.

The noise power depends on the noise characteristics and the bandwidth of $y(t)$, which varies depending on the parameters of the first-order processes that give rise to the signal (k_a , k_b , τ_a , and τ_b). The noise power P_N can be calculated as:

$$P_N = \int_{-\infty}^{\infty} |G(f)|^2 S_n(f) df = \int_{-\infty}^{\infty} |G(f)|^2 \lambda df \quad (7.2)$$

where $G(f)$ is the frequency response of the transfer function $G(s)$, and $S_n(f)$ is the power spectral density of the noise. All the necessary information needed to calculate this power is given by the Identifier Module. Assuming an ideal low-pass filter, and with the disturbances included as filtered white gaussian noise (WGN) (see *Chapter 4 Identifier*), the value of this integral can be approximated to

$$P_N \simeq 2\lambda \cdot BW \quad (7.3)$$

where λ is the variance of the WGN added and BW is the bandwidth of the $G(f)$ which can be estimated from the transfer function $G(s)$ obtained by the Identifier Module.

For our analyses we used equation (7.3) with a bandwidth calculated at a 3 dB drop in gain. A design of a non-ideal low-pass filter with cutoff frequency equal to BW , 1 dB ripple in the pass band, and 60 dB attenuation in the stop band yielded practically identical results.

Figure 7.4: Effect of noise on kinetic parameters and probability of error in classification for the traces in Fig. 7.3. Relative error in transition-rate kinetic parameters (σ_1 , σ_2 , and σ_3) and probability of error in detection for parallel and feedback configurations. Each point in the graph depicts the mean and standard deviation of 100 simulations. Boundary conditions used in the Classifier Module are $\tau_a \in [1, 9]$ ms, $\tau_b \in [50, 250]$ ms, $k_a \in [-20, 20]$, and $k_b \in [-20, 20]$ for the parallel problem and $k_b \in [0, 20]$ for the feedback problem since combinations in feedback with $k_b < 0$ are unstable.

7.2.2 Results

In our tests, traces arising from molecular kinetic schemes associated with the parallel and feedback configurations had similar robustness to noise (Fig. 7.4). The relative errors in the transition rate kinetic parameters began to degrade at a signal-to-noise ratio (SNR) of 47 dB, but remained below a relative error of 0.5 for a SNR = 30 dB and above.

The probability of error in classification increased rapidly for traces with a SNR lower than 25 dB for the parallel configuration and 22 dB for the feedback configuration, reaching a $P_e = 1$ at 18 dB for both configurations. It is worth noting that the P_e represents not only the instances in which a trace in feedback configuration is mistakenly classified as a parallel configuration or vice versa, but also errors in which the trace is classified as arising from a higher-order (≥ 2) or first-order system. These two types of errors quickly dominate the probability of error in detection at high levels of noise as the SNR degrades.

7.3 Improving the SNR requirement for error-free classification

The results depicted on the previous section indicate that, in the presence of additive Gaussian noise, the probability of error in determining the right configuration sharply increased for signal-to-noise ratios below 25 dB and 22 dB for the parallel and feedback configurations, respectively. One possible strategy to reduce the probability of error would be to filter the trace prior to the application of SYSMOLE. Filtering increases the SNR and allows the Identifier Module to successfully detect the poles and zeros, and the Classifier Module to accurately determine the configuration.

To illustrate the use of this strategy, we applied a moving-average filter to the traces generated by the Synthetic Trace Simulator in the main text to study the robustness of SYSMOLE to Gaussian noise in the second-order parallel subtraction and feedback configurations. Filtering the traces resulted in an overall increase in SNR of 6.7 dB and 6.5 dB for the parallel and feedback configurations, respectively (Fig. 7.5, 7.6 and 7.7).

Figure 7.5: Illustration of the improvement in signal-to-noise ratio by filtering. Traces are the result of a parallel subtraction configuration with $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms, and $\tau_b = 100$ ms. Red depicts the trace without noise, cyan the traces with noise, and dark blue the traces filtered with a moving-average filter with a window of 3 ms.

Figure 7.6: Improvement in signal-to-noise ratio (SNR) by filtering the trace with a moving-average filter of window 3 ms prior to application of SYS-MOLE in second-order parallel and feedback configurations.

Figure 7.7: Improvement in probability of error (Perror) in assigning the correct configuration by filtering the trace with a moving-average filter of window 3 ms prior to application of SYSMOLE for the second-order parallel and feedback configurations.

7.4 Other types of Noise

7.4.1 Brownian Noise

In the previous section we explored the effect of added Gaussian noise to the trace on the probability of error of detecting the right configuration from the trace (Fig. 7.4). We decided to test the robustness of SYSMOLE to added Brownian noise, which is common in the diffusion of molecules in anisotropic environments, such as cellular membranes (59, 60). We used the Synthetic Trace Simulator to generate similar traces to those of the L-type calcium and heteromeric GPCR experiments described in *Chapters 9* and *10*, add noise, and test the ability of SYSMOLE to uncover the correct configuration. Specifically, we added Brownian noise with amplitudes ranging from 0.001 to 1.5 to traces generated by two processes with parameters $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms, and $\tau_b = 100$ ms either in feedback or in parallel (Fig. 7.8).

We ran 100 simulations for each level of Brownian noise added, and computed the probability of error as the number of simulations correctly assigned divided by the total number of simulations. The results indicate that SYSMOLE is robust to the presence of Brownian noise in these traces, with probabilities of error starting to increase at a SNR of 18 dB for the parallel configuration and 14 dB for the feedback configuration (Fig. 7.9).

Figure 7.8: Example of traces with added Brownian noise of amplitude 0.25, 0.75, and 1.25 respectively. Traces are the result of a parallel subtraction configuration with $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms, and $\tau_b = 100$ ms. Red depicts the trace without noise.

Figure 7.9: Probability of error in assigning the right configuration by SYS-MOLE as a function of SNR for traces simulated with added Brownian noise. Each data point represents 100 simulations with parameters $k_a = -5$, $k_b = 3$, $\tau_a = 5$ ms and $\tau_b = 100$ ms. Classifier boundary conditions used are $\tau_a \in [1, 10]$ ms, $\tau_b \in [50, 250]$ ms, $k_a \in [-10, 10]$, and $k_b \in [-10, 10]$ for the parallel problem and $k_b \in [0, 10]$ for the feedback problem since combinations in feedback with $k_b < 0$ are unstable

Figure 7.10: Cell-to-cell variability in *Ifnb1* gene induction. (derived from experimental measurements in (64)). Distribution of induction rate (b_a) and gain parameter (k_a), and representative traces for *Ifnb1* induction

7.4.2 Single-cell gene expression noise

In order to explore the versatility of SYSMOLE to tackle non-classical types of noise, we decided to test whether we could use SYSMOLE to tease out different gene regulatory mechanisms in the presence of cell-to-cell gene induction noise. Gene expression in response to a given stimulus varies among cells, even when the cell population is homogeneous (61–63). Our previous studies have successfully utilized single-cell single-molecule techniques to characterize this cell-to-cell variability or noise in the induction of the interferon beta gene (*Ifnb1*), a key cytokine involved in innate immune responses (64). Specifically, it has been established that cell-to-cell variability in the rate of induction (in mRNA molecules per hour) of *Ifnb1* in dendritic cells exposed to a lipopolysaccharide (LPS) present in bacterial walls can be characterized by a gamma distribution with size and shape parameters with values 3 and 2.5 respectively.

In order to translate this finding to our framework, we described gene induction as a first-order process $G_a(s)$ characterized by a time constant $\tau_a = 3$ hours, and a k_a that varies for each cell as follows (Figure 7.10):

$$b_a \in \Gamma(3, 2.5) \quad (7.4)$$

$$k_a = b_a \tau_a \quad (7.5)$$

We included a second first-order process responsible for regulating gene expression in a feedback or parallel subtraction configuration $G_b(s)$, with parameters $k_b = -25$ mRNA mol. for the parallel configuration and 25 mRNA

mol. for the feedback configuration and $\tau_b = 16.67$ hours (1000 min). Molecularly, one could potentially interpret the gene regulatory mechanisms in these schemes in terms of the inhibition of *Ifnb1* expression by a co-expressed gene, or the inhibition of *Ifnb1* by other pathogen factors (65). We then measured the ability of SYSMOLE to recognize the underlying gene regulatory mechanism in the presence of cell-to-cell variability by testing in how many cells, out of 1000, would SYSMOLE obtain the correct configuration. SYSMOLE showed to be robust to cell-to-cell variability of *Ifnb1* induction expression with error probabilities of 0 and 0.0929 for the feedback and the parallel subtraction configurations, respectively (Fig. 7.11)

Similar limitations to those described previously apply for cell-to-cell variability noise. First, sampling frequency should be available to capture the fastest process. Secondly, the gene regulation process should be working at a slower rate than gene induction. Together these results suggest that SYSMOLE is also robust to cell-to-cell variability noise.

Summary and Conclusions

- Our results indicate that for the traces of interest one can perform a noise analysis with similar synthetic traces and establish for a given SNR the expected probability of error in classification and relative error in the transition-rate parameters of the molecular kinetic scheme
- For fast-activation and slow-inactivation macroscopic traces of the types obtained in our experimental systems, L-type calcium channels and G protein-coupled receptor (GPCR) Gi and Gq signaling (see *Chapters 9* and *10*), our results indicated that a SNR of 25 dB or better should provide the right identification of the molecular kinetic scheme and a low relative error in the kinetic parameters.
- Similar robustness to noise of SYSMOLE can be achieved in the presence of other types of noise such as Brownian noise and single-cell noise.
- The robustness to noise can be improved through pre-processing by filtering the signal prior to applying SYSMOLE.

Figure 7.11: Traces and probability of error associated with a potential gene-regulatory mechanism described by a feedback or a parallel subtraction scheme in which cell-to-cell variability noise is included following the noise model described in Figure 7.10. Navy blue traces depict noiseless traces. Additional traces correctly (light blue) or incorrectly (red) assigned to the feedback (top) or parallel subtraction (bottom) configuration are overlaid. Parameters are $\tau_b = 100$ min, $k_b = 25$ for feedback, and $k_b = -25$ for parallel. Classifier parameters are $(\tau_a \in [1, 50], \tau_b \in [75, 250], k_a \in [-10, 10], k_b \in [-100, 100])$ for parallel subtraction and $k_b \in [0, 100]$ for feedback.

Chapter 8

Scalability

Introduction. This chapter will discuss how to scale the Identifier Module, the Classifier Module, and the Molecular Kinetic Converter beyond second order systems.

8.1 Description

To demonstrate the scalability of SYSMOLE, we present in this section its implementation for an example in which the traces arise from a third order system. Let us assume that we have a set of traces in response to a stimulus from 10 different experiments that look like those in Figure 8.1. We would like to use SYSMOLE to extract information about the molecular kinetic scheme / Markov-chain state network underlying these traces.

8.2 Scalability of the Identifier Module

As with real experimental traces, we do not know a priori the order of the system, which is determined by the number of poles and zeros in the transfer function obtained by the Identifier Module. It is indeed difficult to determine merely by visual expression whether these traces are associated with a second-order or a third-order system. The implementation of the Identifier Module does not need scaling since it is already designed to detect as many processes as there may be in the trace. ARX methods have been successfully used to characterize processes of order 5 and up (48, 66). In practice, the real limitation to scalability arises from the sampling frequency of the data and the fastest process that can be captured at that frequency determined by the Nyquist theorem. Furthermore, the presence of noise can result in the loss of some of the poles and zeros. As mentioned in *Chapter 4*, SYSMOLE is

Figure 8.1: Example of 10 simulated experimental traces arising from a third order system. Stimulus was given at time = 30 ms.

best adapted to analyze adequately-sampled traces. *Chapter 7* provides an extensive noise robustness analysis of SYSMOLE and strategies to improve the error-free SNR region.

8.3 Scalability of the Classifier Module

Analogous principles and methodology applied to implement the Classifier Module for second-order systems (as described in the *Chapter 5*) can be adapted for higher-order transfer functions. The flow chart should be expanded and the number of optimization problems increased. In our example, for all ten traces the Identifier Module extracted a third-order transfer function associated with three poles and two zeros. The task of the Classifier Module will be to discriminate among the different combinations of the configurations (cascade, feedback, and parallel) that yield third-order transfer functions with 3 poles and 2 zeros. From all combinations possible, five combinations of configurations can be described with 3 poles and 2 zeros: Cascade-Parallel (CP), Feedback-Feedback (FF), Feedback-Parallel (FP), Parallel-Feedback (PF), and Parallel-Parallel (PP). In a similar fashion to that of second-order systems, one can implement the Classifier Module by solving five optimization problems and comparing the value of their respective cost functions (f_{valCP} , f_{valFF} , f_{valFP} , f_{valPF} , and f_{valPP}) as depicted on Figure 8.2. Furthermore, it should be noted that optimization algorithms,

Figure 8.2: Flow chart of the Classifier Module implementation for third-order systems characterized by two poles and three zeros

such as the one proposed to solve the optimization problems (see *Chapter 5*), have been successfully applied to solve high-order equations (51).

Mathematically, the transfer functions and set of equations to build the cost function in the optimization problem for each combination are as follows:

Cascade-Parallel (CP): 3 poles and 2 zeros

$$G(s)_{CP} = \frac{b_a b_b (s + \omega_c) + b_c (s^2 + 2\omega_a \omega_b s + \omega_a \omega_b)}{(s + \omega_a)(s + \omega_b)(s + \omega_c)} \quad (8.1)$$

This yields for the CP optimization problem:

$$\begin{cases} B_2 = b_c \\ B_1 = b_a b_b + b_c(\omega_a + \omega_b) \\ B_0 = b_a b_b \omega_c + b_c \omega_a \omega_b \\ A_2 = \omega_a + \omega_b + \omega_c \\ A_1 = \omega_a \omega_b + \omega_c(\omega_a + \omega_b) \\ A_0 = \omega_a \omega_b \omega_c \end{cases} \quad (8.2)$$

Feedback-Feedback (FF): 3 poles and 2 zeros

$$G(s)_{FF} = \frac{b_a(s + \omega_b)(s + \omega_c)}{(s + \omega_a)(s + \omega_b + b_b)(s + \omega_c + b_c)} \quad (8.3)$$

This yields for the FF optimization problem:

$$\begin{cases} B_2 = b_a \\ B_1 = b_a(\omega_b + \omega_c) \\ B_0 = b_a \omega_b \omega_c \\ A_2 = \omega_a + \omega_b + b_b + \omega_c + b_c \\ A_1 = \omega_a(\omega_b + b_b) + (\omega_c + b_c)(\omega_a + \omega_b + b_b) \\ A_0 = \omega_a(\omega_b + b_b)(\omega_c + b_c) \end{cases} \quad (8.4)$$

Parallel-Feedback (PF): 3 poles and 2 zeros

$$G(s)_{PF} = \frac{(b_a + b_b)s^2 + s[(b_a + b_b)\omega_c + b_a\omega_b + b_b\omega_a] + \omega_c(b_a\omega_b + b_b\omega_a)}{(s + \omega_a)(s + \omega_b)(s + \omega_c)} \quad (8.5)$$

This yields for the PF optimization problem:

$$\begin{cases} B_2 = b_a + b_b \\ B_1 = b_a\omega_b + b_b\omega_a + b_a\omega_c + b_b\omega_c \\ B_0 = (b_a\omega_b + b_b\omega_a)\omega_c \\ A_2 = \omega_a + \omega_b + b_c + \omega_c \\ A_1 = \omega_a\omega_b + (\omega_c + b_c)(\omega_a + \omega_b) \\ A_0 = \omega_a\omega_b(\omega_c + b_c) \end{cases} \quad (8.6)$$

Parallel-Parallel (PP): 3 poles and 2 zeros

$$G(s)_{PP} = \frac{(b_a + b_b s + b_a \omega_b)s + b_a \omega_b + b_b \omega_a}{(s + \omega_a)(s + \omega_b)} * \frac{b_c}{(s + \omega_c)} \quad (8.7)$$

Param.	CP	FF	FP	PF	PP
k_a	$[-5,5]$	$[-5,5]$	$[-5,5]$	$[-5,5]$	$[-5,5]$
k_b	$[-5,5]$	$[0,5]$	$[0,5]$	$[-5,5]$	$[-5,5]$
k_c	$[-5,5]$	$[0,5]$	$[-5,5]$	$[0,5]$	$[-5,5]$
τ_a	$[2,6]$	$[2,6]$	$[2,6]$	$[2,6]$	$[2,6]$
τ_b	$[30,100]$	$[30,100]$	$[30,100]$	$[30,100]$	$[30,100]$
τ_b	$[30,500]$	$[30,500]$	$[30,500]$	$[30,500]$	$[30,500]$

Table 8.1: Range of parameters for the Classifier Module with three poles and two zeros.

This yields for the PP optimization problem:

$$\begin{cases} B_2 = b_a + b_b + b_c \\ B_1 = b_a\omega_b + b_b\omega_a + (b_a + b_b)\omega_c + b_c(\omega_a + \omega_b) \\ B_0 = \omega_c(b_a\omega_b + b_b\omega_a) + b_c\omega_a\omega_b \\ A_2 = \omega_a + \omega_b + \omega_c \\ A_1 = \omega_a\omega_b + \omega_c(\omega_a + \omega_b) \\ A_0 = \omega_a\omega_b\omega_c \end{cases} \quad (8.8)$$

Once the Classifier implemented, we tested SYSMOLE's ability to determine the right combination of configurations for a wide range of traces. To that aim we generated synthetic traces using each of the five combinations (CP, FF, FP, PF, and PP) to which we added white Gaussian noise with increasing variance. We generated 100 simulations for each noise level, with parameters k_a , k_b , k_c , τ_a , τ_b , and τ_c ranging as described on the Table 8.1. As discussed for the second-order systems (see *Chapter 5*), these ranges were chosen to avoid parameter regions in which solutions for multiple combinations exist. Furthermore, visual inspection of the traces (Fig. 8.1) allows us to determine that the three processes detected by the identifier show differences in their time constants: a fast inflection first, followed by two slower ones. This information has therefore been added to the Classifier Module in the appropriate ranges.

As one could expect, each combination yields signals which vary greatly in terms of the signal power. To compare the robustness to noise among configurations we normalized the signal-to-noise ratio (SNR) of each signal by dividing it by the SNR for that combination to a given level of white Gaussian noise with standard deviation of 10^{-5} . The results reveal a probability of error that increases sharply at a normalized SNR of between 0.5 and 0.6 for

Figure 8.3: Probability of error as a function of normalized SNR in the Classifier Module for combinations with three poles and two zeros. (CP) Cascade-Parallel, (FF) Feedback-Feedback, (FP) Feedback-Parallel, (PF) Parallel-Feedback, and (PP) Parallel-Parallel.

all the combinations (the traces in Figure 8.1 have an equivalent normalized SNR around 0.8). Applying SYSMOLE to traces of our example yielded the combination PF for all ten traces.

8.4 Scalability of the MKC

The analytical methodology described for second-order systems can be extended to higher order systems with more than two processes. To that aim the three canonical second-order configurations (i.e. cascade, feedback, and parallel) can be used as building units to derive the molecular kinetic schemes associated with more complex block diagrams and transfer functions. Examples for third-order systems are provided on Figure 8.4 and Figure 8.5.

In following with our example traces (Fig. 8.1), from the solutions for k_a , k_b , k_c , τ_a , τ_b , and τ_c (Table 8.2) we observe that both k_a and k_b are positive, so that the parallel configuration will be in the addition configuration. For the PF combinations determined by the Classifier Module we would find the molecular kinetic scheme depicted in Figure 8.5. The values for the σ_i can be obtained following the steps described in *Chapter 6* and solving the

Figure 8.4: Parallel/Cascade Third-Order System.

Figure 8.5: Parallel/Feedback Third-Order System.

corresponding system of equations either analytically, or given the increased complexity of the equation systems, using optimization techniques.

The system of differential equations that describes the PF molecular kinetic scheme is:

$$\left\{ \begin{array}{ll} \frac{dz_4(t)}{dt} = \sigma_2 z_1(t) - \sigma_3 z_4(t) & \text{ODE Transition 1} \\ \frac{dz_2(t)}{dt} = \sigma_3 z_4(t) + \sigma_1 z_1(t) + \sigma_5 z_3(t) - \sigma_4 z_2(t) & \text{ODE Transition 2} \\ \frac{dz_3(t)}{dt} = \sigma_4 z_2(t) - \sigma_5 z_3(t) & \text{ODE Transition 3} \\ y(t) = \gamma z_2(t) & \text{Observable Equation} \\ u(t) = z_1(t) + z_2(t) + z_3(t) + z_4(t) & \text{Mass Equation} \end{array} \right. \quad (8.9)$$

We Laplace transform the system using $z_2(0)=0$, $z_3(0)=0$, and $z_4(0)=0$ for flexibility

$$\left\{ \begin{array}{ll} sZ_4(s) = \sigma_2 Z_1(s) - \sigma_3 Z_4(s) & \text{T1} \\ sZ_2(s) = \sigma_3 Z_4(s) + \sigma_1 Z_1(s) + \sigma_5 Z_3(s) - \sigma_4 Z_2(s) & \text{T2} \\ sZ_3(s) = \sigma_4 Z_2(s) - \sigma_5 Z_3(s) & \text{T3} \\ Y(s) = \gamma Z_2(s) & \text{O} \\ U(s) = Z_1(s) + Z_2(s) + Z_3(s) + Z_4(s) & \text{M} \end{array} \right. \quad (8.10)$$

We obtain $G(s)_{kin}$ by isolating $\frac{Y(s)}{U(s)}$ from equations T1, T2, T3, O, and M and simplifying the expression

$$G(s)_{kin} = \frac{\gamma[\sigma_1 s^2 + s[\sigma_3(\sigma_2 + \sigma_1) + \sigma_5 \sigma_1] + \sigma_5 \sigma_3(\sigma_2 + \sigma_1)]}{s^3 + s^2(\sigma_5 + \sigma_4 + \sigma_3 + \sigma_1 + \sigma_4 \sigma_2) + s[\sigma_3(\sigma_1 + \sigma_2 + \sigma_5 \sigma_4) + (\sigma_5 + \sigma_4)(\sigma_1 + \sigma_2)] + (\sigma_2 + \sigma_1)\sigma_3(\sigma_5 + \sigma + 4)} \quad (8.11)$$

We solve for σ_1 , σ_2 , σ_3 , σ_4 , σ_5 and γ computationally by comparing 8.11 to the coefficients B_2 , B_1 , B_0 , A_2 , A_1 , and A_0 of the transfer function $G(s)$ obtained by the Identifier Module (Table 8.2).

Summary and Conclusions

- SYSMOLE can be successfully scaled to higher-order systems.
- The Identifier Module and the MKC Module do not present clear limitations to scalability, and should be easily scaled up to fifth order systems with the burden of the increase in complexity mainly carried by the MKC.

Trace	k_a	k_b	k_c	τ_a	τ_b	τ_c	σ_1	σ_2	σ_3	σ_4	σ_5	γ
1	5.07	2.97	2.92	5.36	48.26	413.49	0.0213	0.9528	0.00033	$2.22 \cdot 10^{-14}$	$2.22 \cdot 10^{-14}$	4.720
2	5.10	2.12	3.39	4.67	48.24	423.02	0.0240	0.7156	0.00045	$2.22 \cdot 10^{-14}$	$2.22 \cdot 10^{-14}$	4.719
3	4.70	2.49	2.57	4.20	46.08	396.11	0.0263	0.7067	0.00052	$2.22 \cdot 10^{-14}$	$2.22 \cdot 10^{-14}$	4.454
4	5.01	2.06	2.94	4.91	52.03	416.04	0.0227	0.6129	0.00045	$4.29 \cdot 10^{-14}$	$1.81 \cdot 10^{-14}$	4.650
5	4.94	2.19	3.48	5.71	45.08	417.10	0.0204	0.7841	0.00035	$3.22 \cdot 10^{-14}$	$1.00 \cdot 10^{-14}$	4.465
6	5.32	2.16	2.53	5.65	52.79	376.25	0.0202	0.9999	0.00026	$2.22 \cdot 10^{-14}$	$2.22 \cdot 10^{-14}$	4.847
7	5.00	2.26	2.57	5.08	51.06	423.85	0.0220	0.7148	0.00039	$2.22 \cdot 10^{-14}$	$2.22 \cdot 10^{-14}$	4.653
8	4.54	2.42	3.34	5.46	46.58	386.01	0.0210	0.5387	0.00050	$2.28 \cdot 10^{-14}$	$1.69 \cdot 10^{-14}$	4.192
9	4.80	2.55	2.59	5.43	45.37	395.72	0.0210	0.6294	0.00047	$2.83 \cdot 10^{-14}$	$9.04 \cdot 10^{-14}$	4.669
10	5.29	2.81	3.40	5.40	49.18	386.50	0.0213	1	0.00031	$2.23 \cdot 10^{-14}$	$2.23 \cdot 10^{-14}$	4.851

Table 8.2: List of parameters obtained for 10 simulated experimental traces arising from a third order system with two poles and three zeros.

- The Classifier Module represents the bottle-neck in terms of scalability, as the number of optimization problems to solve increases and the boundary conditions for these problems need to be carefully selected to avoid overlap. A successful application to third order systems is documented in our results.
- In practice, many questions regarding biological systems refer to second and third order problems, which makes this method suited to be applied to a wide range of macroscopic traces.

Chapter 9

Application of SYSMOLE to study the mechanism of action of nifedipine

Introduction. In this chapter we will demonstrate the applicability of SYSMOLE to experimental traces. We will study macroscopic electrophysiological calcium channel current traces, and test whether SYSMOLE can correctly identify the mechanism of action of nifedipine, a calcium channel blocker used in the clinic.

9.1 L-type Calcium Channels and Nifedipine

9.1.1 Ion channels

Ion channels are integral membrane proteins that span the cell membrane (lipid bilayer) providing a pathway for ions to cross the membrane. Phospholipids form self-sealing lipid bilayers that are the basis for cellular membranes. Phospholipids have a hydrophilic head and a hydrophobic tail. The hydrophobic tails join to exclude water and ions, while the polar hydrophilic heads face the aqueous environment of the extracellular fluid and cytoplasm (Fig. 9.1).

Ions in solution are surrounded by a cloud of water molecules (Fig. 9.1 left enlargement) that are attached to the net charge of the ion. This cloud is carried along by the ion as it diffuses through solution, increasing the effective size of the ion. It is energetically unfavorable, and therefore improbable, for the ion to leave this polar environment to enter the nonpolar environment

of the lipid bilayer. The illustration in Figure 9.1 depicts how a positively charged ion (red) attracts the electronegative oxygen atoms of the surrounding water molecules.

Ion channels possess three important properties: (i) they conduct ions, (ii) they recognize and select specific ions, and (iii) they open and close in response to specific electrical, chemical, or mechanical signals. The channels in nerve and muscle conduct ions across the cell membrane at extremely rapid rates, thereby providing a large flow of ionic current (up to 100 million ions may pass through a single channel per second) (Fig. 9.1). This current flow causes rapid changes in membrane potential which are required for signaling and are critical for axon potential propagation in the nervous system, and contraction of heart and skeletal muscle.

Despite their ability to conduct ions at high rates, ion channels are surprisingly selective: each type allows only one or a few types of ions to pass. A key to the great versatility achieved by neurons and muscle cells is the activation of different classes of ion channels selective for specific ions during action potentials. For example, the membrane potential of nerve cells at rest is largely determined by channels that are selectively permeable to potassium (K^+). Typically, these channels are 100-fold more permeable to K^+ than to sodium (Na^+). Although a Na^+ ion itself is smaller than a K^+ ion, its effective diameter in solution is larger because its local field strength is more intense causing it to attract a larger cloud of water molecules. Thus, these channels can select for K^+ over Na^+ by excluding hydrated ions whose diameter is larger than the pore. Other channels like Na^+ channels have a selectivity filter somewhere along the length of the channel, with a site that weakly binds the permeable ions. According to the hypothesis developed by Bertil Hille and colleagues (12), in sodium channels a Na^+ ion binds transiently at an active site as it moves through the filter. At the binding site the positive charge of the ion is stabilized by a negatively charged amino acid residue on the channel wall and also by a water molecule that is attracted to a second polar amino acid residue on the other side of the channel wall (see enlargement in Fig. 9.1). It is thought that a K^+ ion, because of its large diameter, cannot be stabilized as effectively by the negative charge and therefore will be excluded from the filter.

Finally, many channels are regulated or gated, i.e. they open and close in response to various stimuli, and as such are most sensitive to one type of signal. *Voltage-gated channels* are regulated by changes in voltage, *ligand-gated channels* by chemical transmitters, and *mechanically-gated* channels by

Figure 9.1: The ionic permeability properties of the membrane are determined by the interactions of ions with water, the membrane lipid bilayer, and ion channels. A model showing how ion channels are able to select for either K^+ or Na^+ ions. Adapted from (67).

pressure or stretch. In addition to the gated channels, there are non-gated channels which are normally open in the cell at rest. These *resting channels* contribute significantly to the resting potential. For this application of SYSMOLE we will focus on voltage-gated calcium channels.

9.1.2 Voltage-gated ion channels

The voltage-gated ion channels constitute a large family of heteromultimeric membrane proteins whose functional conformations are sensitive to changes in the electrical potential across the cell membrane. They participate in cellular signaling by permitting the flow of cations down an electrochemical gradient through their pore formation subunits. Voltage-gated Na^+ , Ca^{2+} , and K^+ channels share a common architecture and a common set of voltage-dependent functionally distinct states: closed or resting channels, activated or open channels, and inactivated channels (for review see (12) Fig. 9.2). Upon depolarization, these channels change from resting to activated states within milliseconds and then inactivate rapidly. Repolarization is necessary to return to the resting state again. These channels differ in their selectivity for permeant cations as mentioned above, but also in the kinetics and voltage dependence of their transitions among the closed, open, and inactivated states. Voltage-gated Na^+ and K^+ channels also differ from voltage-gated Ca^{2+} in the physiological functions linked to the channel opening. While the flow of Na^+ and K^+ across cell membranes is mainly linked to alterations in membrane potential, the entry of Ca^{2+} into cells through voltage-gated Ca^{2+} channels is coupled to many cellular responses, ranging from neurosecretion and muscle contraction to gene expression. In summary, Ca^{2+} channels transduce electrical signals in the cell membrane into physiologically responses.

9.1.3 L-type calcium ion channels and calcium channel blockers

Several types of voltage-gated Ca^{2+} channels have been identified electrophysiologically and genetically and have been designated L, N, P/Q, R and T (for a review, see (12)). All L-type channels share a common pharmacological profile with high sensitivity to several classes of drugs that have been used as selective L-type channel blockers. The three main classes of L-type-selective Ca^{2+} channel blockers currently available for clinical use are the phenylalkylamines, the benzothiazepines, and the dihydropyridines. These

Figure 9.2: Conserved pseudotetrameric domain structure of voltage-gated channels. Each of the four homologous repeats contributes one fourth to the channel pore and contains a functional voltage sensing domain (marked with + charges) in its fourth transmembrane helix. Adapted from (68).

drugs bind to three separate ion-channel sites on L-type Ca^{2+} channels.

The inactivation mechanism of L-type channels has been extensively studied and two major inactivation molecular mechanisms have been identified and characterized: a fast calcium-dependent inactivation mediated by the calcium ions entering inside the cells upon opening of the channel, and a voltage-dependent inactivation mediated by the voltage sensor located in the principal subunit and its movement upon depolarization to block the channel (Fig. 9.3 and see (12, 69) for a review).

L-type calcium channels are distinguished by their sensitivity to 1,4-dihydropyridine (DHP) calcium channel agonists and antagonists (69–72). Nifedipine is a calcium DHP-antagonist channel blocker widely used clinically as an antianginal and antihypertensive drug (73, 74). Nifedipine, as part of the dihydropyridine class of channel blockers, binds to specific regions of the principal subunit to block the channel. The affinities of nifedipine for these binding regions depend on the state of the channel: nifedipine has a high affinity site for the inactivated state, a low affinity site accessible in the open state, and virtually no affinity site for the closed state. In other words, nifedipine can only access and block the calcium channel once the channel is open (Fig. 9.3).

Figure 9.3: Inactivation mechanisms of calcium channels and mechanism of action of nifedipine.

9.2 Experimental System and Electrophysiological Traces

We used macroscopic current traces from voltage clamp (VC) experiments performed in the accessory radula closer (ARC) muscle of *Aplysia californica* previously obtained (75), to illustrate the potential of SYSMOLE to retrospectively analyze macroscopic traces acquired in previous experiments.

The ARC muscle and its innervation is a model preparation for the study of the neural and cellular mechanisms of modulation of muscle contractions by a variety of neurotransmitters. The main membrane ion currents present and involved in the contraction of the unmodulated ARC muscle are: i) two currents prominent at hyperpolarized voltages (a classical inwardly rectifying K^+ current and a chloride (Cl^-) current induced by elevated intracellular Cl^-), ii) two large outward K^+ currents activated at more depolarized voltages, and iii) an underlying inward depolarization-activated Ca^{2+} current that is normally completely masked by the K^+ currents and is revealed when they are blocked. Being Ca^{2+} a key component of muscle contraction, studying these currents is key to understand the effect of different drugs on muscle function.

The resting membrane potential in muscle is between -90 to -70 mV. The Ca^{2+} current begins to activate as the cell depolarizes around -40 or -30 mV. It activates in milliseconds, then inactivates relatively slowly with maintained depolarization. This inward influx of Ca^{2+} through this current most likely provides the Ca^{2+} necessary for contraction of the ARC muscle.

We recorded currents in response to a depolarization step from -90 mV to 0 mV. We isolated the current through the L-type calcium channels from potassium currents by adding selective blockers (tetraethylammonium and 4-aminopyridine). Calcium ions were replaced by barium in the solutions to remove the contribution of calcium-dependent inactivation and isolate that of the voltage-dependent inactivation of the channels. The currents were measured in the absence or presence of nifedipine (100 nM and $1\mu M$) (see Figure 9.4 for representative current traces).

Figure 9.4: Representative L-type calcium channel current (output) elicited by a voltage step from -90 mV to 0 mV (input), in the absence and presence of nifedipine at 100 nM and 1 μ M concentrations.

9.2.1 Voltage clamp measurements

Reliable techniques for recording the electrical potential across cell membranes were developed in the late 1940s. These techniques allow accurate recordings of both the resting and the action potentials and make use of glass micropipettes filled with a concentrated salt solution that serve as electrodes. These microelectrodes are placed on either side of the cell membrane. Wires inserted into the back ends of the pipettes are connected via an amplifier to an oscilloscope, which displays the amplitude of the membrane potential in volts. Because the tip diameter of a microelectrode is very small ($<1\mu\text{m}$), it can be inserted into a cell with relatively little damage to the cell membrane (Fig. 9.5 A).

When both electrodes are outside the cell no electrical potential difference is recorded. But as soon as one microelectrode is inserted into the cell the oscilloscope shows a steady voltage, the resting membrane potential. In most nerve cells at rest, the membrane resting potential is around -65 mV (Fig 9.5 B). The membrane potential can be experimentally changed using a current generator connected to a second pair of electrodes (one intracellular and one extracellular). When the intracellular electrode is made positive with respect

to the extracellular one, a pulse of positive current from the current generator will cause current to flow into the cell from the intracellular electrode. This current returns to the extracellular electrode by flowing outward across the membrane. As a result, the inside of the membrane becomes more positive while the outside of the membrane becomes more negative. This progressive *decrease* in the normal separation of charge is called *depolarization* (Fig. 9.5 C).

Small depolarizing current pulses evoke purely electronic (passive) potentials in the cell in which the size of the change in potential is proportional to the size of current pulses. However, sufficiently large depolarizing current triggers the opening of voltage-gated ion channels. In voltage clamp (VC) experiments the voltage is held constant while the current flowing through the ion channels in the cell membrane is measured based on the amount of current that an amplifier needs to supply to maintain the set voltage (Fig. 9.5D). In the electrophysiological traces we had of L-type calcium channels, the input voltage had also been measured. We used the voltage as a function of time $V(t)$ (a step from -90 to 0 mV) as the input trace, and the recorded current $i(t)$ as the output trace for SYSMOLE.

9.3 Hypothesis

We tested whether we could derive molecular kinetic schemes consistent with the known molecular mechanism of voltage-dependent inactivation and nifedipine blockade of the L-type channels from experimental macroscopic current traces utilizing SYSMOLE.

9.4 Results

Following the noise analysis described in (*Chapter 7*), the SNR of all experimental traces (>38 dB for all cases) predicted a low probability of error in the identification by SYSMOLE of the correct molecular kinetic scheme as well as a low relative error for the transition rate parameters σ_k (Fig. 9.4 and Table 9.1)

In biological terms, we can explain the current trace $i(t)$ elicited by a step in voltage in a VC experiment using a molecular kinetic scheme as follows.

Figure 9.5: Voltage clamp measurements. (A) The recording setup, (B) oscilloscope display, (C) depolarization, and (D) hyperpolarization. Adapted from (67).

[Nif.]	SNR(dB)	Config.	σ_1	σ_2	σ_3	γ
0 nM	47.30 $\pm$ 5.92	f(100%)	0.48 $\pm$ 0.01	3x10 ⁻³ $\pm$ 7.95x10 ⁻⁴	1.18x10 ⁻³ $\pm$ 2.94x10 ⁻⁴	-2.41 $\pm$ 0.14
100 nM	51.98 $\pm$ 0.89	f(100%)	0.45 $\pm$ 0.03	4.4x10 ⁻³ $\pm$ 2.73x10 ⁻⁴	9.32x10 ⁻⁴ $\pm$ 2.35x10 ⁻⁴	-2.14 $\pm$ 0.05
1 μ M	42.82 $\pm$ 3.57	f(100%)	0.39 $\pm$ 0.07	2.35x10 ⁻² $\pm$ 2.25x10 ⁻²	6.76x10 ⁻⁴ $\pm$ 8.73x10 ⁻⁴	-2.56 $\pm$ 1.18

Table 9.1: Kinetic Parameters Ion-channel Traces. N= 3 - 8 traces per group. f(100%) indicates feedback configuration was obtained for 100% of the traces

Initially, the system is at steady-state and the fractional occupancies (i.e. the fraction of channels in any particular state) of every state are constant and the population of channels in dynamic equilibrium. At this point in time, the current readout is also constant (basal current) and proportional to the fractional occupancy in the open state. When the input steps to a higher voltage, the rate constants which govern the transitions between the states change and the population of channels redistributes accordingly among the states. This redistribution is accompanied by a transient in the fractional occupancies, and thus in the current recorded. At the end of this transient a new steady-state level of current is reached. The value of the rate constants characterizing the transitions before the step in voltage are unknown, and therefore, the σ_k strictly represent the change in rate associated with the input, in this case a step in voltage.

The results of applying SYSMOLE to derive the kinetic schemes from macroscopic traces of L-type calcium channel currents obtained in the voltage-clamp (VC) experiments described, consistently yielded the molecular kinetic scheme associated with a feedback configuration (Fig 9.6 and Table 9.1). Based on the known mechanism of action of nifedipine (76), and due to the high affinity binding of nifedipine to the inactivated state, we expected the presence of this drug to reduce the transition rate from the inactivated state to the open state (I $\rightarrow$ O). Similarly, due to the existence of a binding site for nifedipine in the open state of the channel, we also expected and increase in the transition rate between the open state and the inactivated state (O $\rightarrow$ I), which now includes two different states: the one reached through voltage-dependent inactivation, and the inactivation due to the presence of nifedipine. Finally, we expected nifedipine not to affect the transition between the closed and the open state (C $\rightarrow$ O) since it does not bind to the channel in the resting closed state. All these effects were extracted by SYSMOLE as detailed in Table 9.1, and illustrated by the temporal evolution of the fractional occupancies in the extracted molecular kinetic scheme (Fig. 9.6 and Fig. 9.7)

Figure 9.6: Molecular kinetic scheme obtained by applying SYSMOLE to the traces and expected effects of nifedipine on transition rates.

Figure 9.7: Fractional occupancies as a function of time of the closed-channel (black), open-channel (red), and inactivated-channel (blue) states resulting from stimulating the molecular kinetic scheme. Simulations were generated with the mean value of the kinetic parameters ($N = 3-6$ traces per treatment). Kinetic parameter values can be found on Table 9.1.

Summary and Conclusions

- SYSMOLE performed strongly with experimental traces.
- Application of SYSMOLE to L-type channel traces treated with nifedipine allowed us to derive the correct molecular kinetic scheme of the voltage-dependent inactivation of the L-type calcium channels and retrieve the molecular mechanism of action of nifedipine.

Chapter 10

Application of SYSMOLE to study heteromeric G protein-coupled receptor complexes

Introduction. In this chapter we will apply SYSMOLE to differentiate between *cis*- and *trans*-activation in heteromeric G protein-coupled receptor complexes and generate hypotheses regarding the mechanism of action of glutamate antipsychotics.

10.1 GPCRs and G protein Signaling

In order to achieve physiological regulation, multicellular organisms require a wide variety of cellular types with specialized functions to respond differently to external stimuli. Among the extracellular mediators that modulate and coordinate the cell's activity are photons, the chemical compounds responsible for taste and the sense of smell, peptides, ions, lipids, hormones, and neurotransmitters. Specialized proteins on the cell membrane called receptors detect the presence or absence of these mediators and convey this information inside the cell.

Based on their cell-signaling mechanisms, all the receptors in the cell that recognize neurotransmitters fall into three main groups: extracellular ligand-gated ion channels, enzyme-linked receptors (e.g. insulin receptor family), and G protein-coupled receptors (GPCRs), which are the focus of the application of SYSMOLE described in this chapter. GPCRs are proteins that

span the cell membrane and specialize in transducing extracellular cues into intracellular signals. The GPCR superfamily is comprised of over 800 genes (approximately 4% of the genome). Based on their ability to bind specific ligands (i.e. the adrenergic receptors selectively bind to adrenaline and noradrenaline), the GPCRs are grouped into receptor subtypes, and based on their amino acid sequence homology, the GPCR superfamily is divided into receptor classes (e.g. class A, class B, class C). Despite the lack of sequence homology between classes, all GPCRs share a common structure and a common signaling transduction mechanism.

The signaling mechanism of GPCRs involves the direct interaction with a group of heterotrimeric membrane-bound proteins (G proteins) that bind guanine nucleotides. This is a mechanism highly conserved throughout evolution and found in many species. Based on the structural conformation of the receptor to which they are coupled, upon ligand binding GPCRs convey intracellularly to G proteins the signal they receive. Receptor activation by endogenous ligands (e.g. hormones, neurotransmitters) or by therapeutic drugs triggers the dissociation of G proteins in the membrane into two of its constitutive parts ($G\alpha$ and $G\beta\gamma$). G proteins can be seen to be acting as switches between two states: an inactive state in which the α subunit, bound to guanosine diphosphate (GDP), and the $\beta\gamma$ dimer form a heterotrimer, and an active state in which the α subunit, bound to guanosine triphosphate (GTP), is dissociated from the $\beta\gamma$ dimer. These $G\alpha$ and $G\beta\gamma$ components activate a series of effectors and generate a characteristic cascade of events in the cell.

There are three main subtypes of G proteins ($G_{i/o}$, G_s , and $G_{q/11}$). Each subtype is associated with specific events in the cell. From a systems biology perspective it is important to understand that these events occur in the cell at different time scales. For example, the activation of G protein sensitive potassium channels (GIRK) by the $\beta\gamma$ subunits liberated upon dissociation of G_i subunits ($G\beta\gamma_i$) occurs in less than a second (77), while the induction of the *egr-2* gene due to activation of transcription factors downstream of G_i activation (78) can take minutes to hours. Furthermore, different G protein subtypes might converge at the same effector and have opposing effects at similar or different time scales (e.g., $G_{q/11}$ takes tens of seconds to inhibit currents through GIRK channels via phosphatidylinositol 4,5 bisphosphate (PIP_2) hydrolysis in the membrane, while $G_{i/o}$ activates these channels in less than a second (79)).

GPCRs are not only expressed in nervous tissue but virtually in every

tissue, where they are involved in a wide variety of processes that range from contraction of the arteries to immune responses. These receptors are the target of approximately 700 drugs, which represent 35% of all approved drugs currently in use (80). Not surprisingly, much effort is directed towards new drug discovery targeting GPCRs and furthering our understanding regarding their signaling properties.

10.2 GPCR Heteromerization

10.2.1 Challenging the classic paradigm

In the classic paradigm one molecule of agonist activates one GPCR, which in turn stimulates one subtype of G protein inside the cell. Indeed, biophysical studies have shown that upon agonist binding, monomers (one single receptor) of class A GPCRs (e.g. β_2 -adrenergic receptor, rhodopsin, μ -opioid receptors), when reconstituted in lipid vesicles, couple and activate their respective G proteins (81–83). Also monomeric rhodopsin in solution can activate its G protein transducin (84).

However, we now have significant amount of experimental data that challenges the classic paradigm and indicates that GPCR oligomerization, both homo and heteromerization, is a general phenomenon. Increasing evidence has shown that GPCRs form complexes of the same GPCR molecule (homomers) and that these homomers have different signaling properties than those of the monomers. In addition, complexes formed by two different GPCRs (heteromers) have been found recently, adding yet another layer of complexity to the signaling capabilities of GPCRs (see (85) for a review on homomers and heteromers). These new findings increase the dimension of the signaling space of GPCRs, presented in such a linear fashion by the classic paradigm, and raise the question of what the physiological role of heteromers in the cell might be, and furthermore, of how their signaling properties affect our understanding of drug effects.

A new vision in the GPCR oligomer field is emerging suggesting that homomers and heteromers have defined and distinct physiological roles in the cell. Consequently, disruption or dysregulation of heteromers and subsequent loss of function might lead to pathophysiological states, which could be helped by heteromer-targeted pharmacology. However, we are only beginning to understand the role of GPCR heteromerization in the cell.

10.2.2 Special characteristics of GPCR heteromers

A receptor heteromer has been defined as “a macromolecular complex composed of at least two functional receptor units with biochemical properties that are demonstrably different from those of its individual components” (85). Similarly, a homomer is the same as a receptor heteromer, but combining two or more identical (functional) receptor units. Oligomerization confers unique pharmacological properties on these receptors, some of which are common to heteromers and homomers. The pharmacological changes upon oligomerization fall into four categories: a) allosteric modulation, b) changes in ligand recognition, c) changes in G protein coupling, and d) changes in trafficking. Understanding these changes is crucial to develop new therapies, and there is a need for new tools to analyze their signaling properties.

10.3 Heteromers and Disease

Heteromers appear to be involved in many diseases and have been described as potential targets for new drugs. On the top of the list we find:

- the heteromers formed by the β_2 -adrenergic receptor together with the angiotensin receptor 1 and the (β_2 -AR/AT1R) or the β_1 -adrenergic receptor (β_2 -AR/ β_1 -AR), which have been linked to cardiac contractility in myocytes establishing their relevance in arrhythmias (86, 87)
- the angiotensin receptor 1 and bradykinin receptor 2 (AT1R/B2R) heteromer which has been shown to have a role in pre-eclampsia (88)
- the prostaglandin E receptor 1 and β_2 -adrenergic receptor (EP1/ β_2 -AR) heteromer, which has been connected to asthma (89)
- the apelin receptor and angiotensin receptor 1 (APJ/AT1R), which is believed to be involved in the formation of the atherosclerotic plaque (90)
- the adenosine 2A receptor and dopamine receptor 2 (A2A/D2R) heteromer that has been connected to Parkinson's disease (85)
- and finally, the heteromer formed by the metabotropic glutamate receptor 2 (mGluR2) and the serotonin receptor 2A (2AR) which is the

focus of this chapter and whose dysregulation is thought to predispose to schizophrenia (91).

New pharmacological approaches that take heteromerization into account are therefore needed. As mentioned before, although these new approaches appear extremely promising, they require as a first step a deep understanding of the signaling properties of each heteromer. The current chapter focuses on the heteromer formed by the mGluR2/2AR and its signaling. Namely, we study the changes in G protein coupling that result from the heteromerization, and explore how SYSMOLE can be used to obtain information regarding the mechanism of action of glutamate antipsychotic drugs that target this receptor.

10.4 The mGluR2/2AR Heteromer

The heteromer formed by the metabotropic glutamate receptor (mgluR2/2AR) has been described to be implicated in psychosis and schizophrenia (91–93). The interaction between the two receptors to form a heterocomplex was established using different experimental biochemical assays. In addition, mGluR2 and 2AR have been shown to co-express and co-localize in mouse layer V pyramidal neurons from the somatosensory cortex and mouse cortex primary cultures.

The mGluR2/2AR complex formation is specific, since the metabotropic glutamate receptor 3 (mGluR3), a receptor highly homologous to mGluR2, and the serotonin receptor 2C (2CR), a receptor highly homologous to 2AR, were unable to form a complex. This specificity has been shown through pharmacological, biochemical, and biophysical assays (91).

The mGluR2/2AR heteromer is unique in that it is the first described heteromer formed by two receptors belonging to two different classes of GPCRs (non-homologous receptors): mGluR2 is a class C receptor similar to pheromone receptors, while 2AR is a class A receptor similar to the rhodopsin, a receptor expressed in the retina that is activated by light. The formation of GPCR complexes across different receptor classes further emphasizes the extent and importance of heteromerization as a general mechanism to achieve and regulate diverse signaling in cells.

Figure 10.1: The mGluR2/2AR GPCR heteromeric complexes and drugs known to target each receptor.

10.5 The mGluR2/2AR and Schizophrenia

Schizophrenia is a psychiatric disorder which affects 1% of the population (94, 95), with an onset in adolescence or early adulthood. Symptoms fall mainly into three categories: positive symptoms, negative symptoms, and cognitive problems. Positive symptoms include hallucinations, in which the patient hears or sees things that are not present, and delusions, commonly resulting from a failure of the patient to adequately interpret his or her perceptions and experiences. Negative symptoms are related to blunted affect, poor language, fatigue, and apathy. Finally, cognitive disorders in schizophrenic patients are typically represented by an inability to concentrate and focus their attention.

Treatment of schizophrenia focuses on treating the symptoms but not the causes of psychosis, which remain elusive to date. In most cases, these disabling symptoms persist for life, and antipsychotic medications currently available are often only partially successful and associated with severe side effects (e.g. parkinsonism, extrapyramidal symptoms, muscular dystonia, weight gain, and decrease in white-blood cell count). Furthermore, some patients remain unresponsive to therapy, and suicide is still the leading cause of premature death in schizophrenic patients (96, 97).

Neuropathological evidence involves several neuronal circuits in schizophrenia, all of which are responsible for processing sensory stimuli (98, 99). In order to process sensory information, cortical pyramidal neurons need to inte-

grate the neuronal input of projections from different nuclei mediated through different neurotransmitters: glutamate directly from the thalamus and via interneurons, dopamine directly from the ventral tegmental area or via interneurons, serotonin from the raphe nuclei, and noradrenaline from the locus coeruleus. Our work will focus on the integration of the signaling mediated by serotonergic and glutamatergic projections through the mGluR2/2AR heteromer (Fig. 10.2).

Due to the similarity between some of the symptoms observed in these patients and those elicited by hallucinogens, schizophrenia research has progressed in parallel with the new discoveries in the mechanism of action of hallucinogens and antipsychotic drugs.

10.5.1 Psychedelics are hallucinogens that target 2AR

Generally speaking, ligands can be classified into three main categories based on their effect on GPCR signaling. Agonists are ligands that bind to the GPCR and stabilize an active conformation of the receptor resulting in an increase of signaling through the G protein-subtype pathway associated with that receptor. Inverse agonists bind and stabilize the GPCR in an inactive conformational state (100). Finally, neutral antagonists (101–103), compete for the same orthosteric binding site as the agonist and prevent the cellular responses induced by agonists and inverse agonists.

Phencyclidine (PCP), psilocybin, mescaline and lysergic acid diethylamide (LSD) are described as hallucinogens based on the effects they induce in perception, cognition, sensory processing, self-representation and changes in brain metabolism. Hallucinogens can be divided into two main groups: dissociative drugs, and psychedelics. Dissociative PCP-like drugs (e.g., ketamine and MK801) are non-competitive antagonists at glutamate *N*-methyl-*D*-aspartate (NMDA) receptors (104). Psychedelic drugs such as LSD, mescaline, and psilocybin primarily act as agonists on the 2AR receptors, as demonstrated in recent findings showing that inactivation of serotonin 2AR signaling by either genetic or pharmacological approaches results in markedly reduced behavioral responses to hallucinogenic drugs in both rodent models (78, 105, 106) and humans (107). Thus, although psychedelics bind also to other receptor subtypes, the 2AR receptor is considered necessary for the unique behavioral activity of these chemicals.

Figure 10.2: Neuronal circuits implicated in the responses induced by psychoactive chemicals and antipsychotic drugs. **(A)** The serotonin and glutamate release induced in the cortex by PCP-like psychoactive drugs targeting subcortical NMDA receptors might affect the signaling properties of the cortical mGluR2/2AR receptor complex. **(B)** Concurrently, both LSD-like and PCP-like psychoactive drugs dysregulate the signaling properties of cortical pyramidal neurons and affect cognition and perception processes in the brain cortex.

10.5.2 Atypical antipsychotics target the 2AR

The first antipsychotic, chlorpromazine was serendipitously discovered in 1952 by Jean Delay while investigating anti-inflammatory drugs for post-operative traumatic shock. Jean Delay also coined the term *neuroleptic* for drugs that are able to mitigate psychotic symptoms. Two of the most widely used antipsychotic drugs (haloperidol and clozapine) were also found as an unexpected result of different therapeutic endeavors: haloperidol was being developed as an analgesic, and clozapine was described as an antidepressant with neuroleptic properties. Thus, not surprisingly, most antipsychotics share pharmacological profiles similar to those of clozapine or haloperidol. Antipsychotic drugs have a high affinity for monoaminergic receptors and share an antiserotonergic and antidopaminergic pharmacological profile. Based on their relative affinity for different receptors, antipsychotics can be classified into first generation or typical, and second generation or atypical. Typical antipsychotics are similar to haloperidol and bind with higher affinity to dopamine receptors (namely to the D2R) than to 2AR, while second generation or atypical antipsychotics are similar to clozapine and bind 2AR with higher affinity than dopamine receptors. First generation antipsychotics treat effectively only positive symptoms and present severe side effects (e.g. parkinsonism and muscular dystonia), which led to their replacement by the atypical antipsychotics. These second-generation antipsychotics are effective in treating both positive and negative symptoms and have less side effects in short-term treatment (see Table 10.1). Since the mid-nineties no drugs with new targets and effective antipsychotic properties have been approved, and atypical antipsychotics remain the first-line of treatment for schizophrenia. Unfortunately, between 10% and 30% of patients with schizophrenia have been reported to have little or no response to antipsychotic medication (108) and 60-70% improve with antipsychotic medication but do not achieve complete remission (109–111).

The atypical antipsychotics clozapine, olanzapine, quetiapine and risperidone are the most commonly used drugs for treating schizophrenia. Given that all atypical antipsychotics have a high affinity for the same subtype of serotonin receptor (2AR), and that clozapine is able to block both the biochemical and the behavioral effects induced by hallucinogenic drugs, the current vision is that the serotonin 2AR expressed in pyramidal neurons is in part responsible for the therapeutic effects of the drugs used in the treatment of schizophrenia (Fig. 10.2).

However, a fundamental question remains regarding the mechanism of

	Classification	Repres. Drugs	Mechanism	Efficacy	Side Effects
50s	Typical (1 st Gen.)	Chlorpromazine, haloperidol	D2R block	Positive symptoms. Not efficient against negative symptoms.	Parkinsonism, muscular dystonia
90s	Atypical (2 nd Gen.)	Clozapine	2AR block	Positive symptoms. Some negative symptoms. Most efficient drug for treatment-resistant schizophrenia	Reduced Parkinsonism Severe side effects in some patients (agranulocytosis)
1995	New atypical	Olanzapine	Similar to clozapine	Positive symptoms. Some negative symptoms. Less efficient for treatment-resistant schizophrenia	Reduced side effects

Table 10.1: A History of Drugs for Treating Schizophrenia.

action of both antipsychotic drugs and hallucinogens. All atypical antipsychotic drugs share a high affinity for 2AR and block most of its functional responses; however, not all the drugs that block the 2AR are antipsychotics. In a similar fashion, all hallucinogenic drugs bind and activate 2AR, but not all the drugs that bind 2AR are hallucinogens (e.g., lisuride and ergotamine used for Parkinson's Disease and migraine do not induce hallucinations or delusions). The molecular determinants for a drug to behave as an atypical antipsychotic are currently poorly understood, but it has been recognized that most clinically effective antipsychotic drugs are, in fact, 2AR inverse agonists, rather than simply neutral antagonists (102).

10.5.3 Glutamate antipsychotics

In September 2007, new clinical findings were published that opened a new potential approach for the treatment of schizophrenia. It was found that treatment of schizophrenic patients with LY404039, a potent and selective mGluR2 agonist, reduced both positive and negative symptoms. In contrast with antipsychotics (atypical and classic), this improvement did not present the classic side effects associated with drugs with high affinity for 2AR and D2R such as tardive dyskinesia and parkinsonism. These results, albeit encouraging from a clinical point of view, gave rise to new questions regarding the neurochemical basis of schizophrenia, which presently includes:

- the dopamine hypothesis, inspired by the capacity of the first generation antipsychotics to block D2R receptors
- the serotonin hypothesis, based on the fact that both psychedelics and atypical antipsychotics target 2AR receptors
- the glutamate hypothesis, which includes the antagonism at NMDA receptors and the newly-found agonism at mGluR2/3 receptors.

Metabotropic glutamate receptors mGluR2/3 have been the target of considerable attention regarding the molecular mechanism underlying psychosis. The recent identification of the heteromer formed by mGluR2/2AR in mouse and human brain has become the starting point of the convergence of both the serotonin and the glutamate hypotheses of schizophrenia. Functionally, it was found that activation of mGluR2 inhibits the cellular and behavioral responses induced by hallucinogenic drugs (112), suggesting the direct implication of mGluR2/2AR in the mechanism of action of glutamate antipsychotics. Moreover, in post-mortem human brain from untreated schizophrenic patients, 2AR expression has been shown to be up-regulated while mGluR2 expression is down-regulated (91), suggesting that these regulatory changes may be involved in the altered cortical processes of schizophrenia.

10.6 Questions and Hypothesis

The dysregulation of the heteromeric complex formed by the Gi-coupled metabotropic glutamate receptor 2 (mGluR2) and the Gq-coupled serotonin 2A receptor (2AR) has been linked to schizophrenia (91). Preclinical and clinical studies suggest that activation of mGluR2 in the complex by allosteric and orthosteric agonists could represent a new therapeutic approach to treat schizophrenia and other disorders (64, 113, 114). Recently published work shows that psychedelic drugs upset the Gi/Gq signaling balance associated with the mGluR2/2AR complex by reducing Gi and increasing Gq signaling, while antipsychotic drugs restore the natural Gi/Gq balance. This is achieved through a crosstalk mechanism in which a ligand acting on one of the receptors, either 2AR or mGluR2, can alter the signaling on their counterpart receptor (115). However, the mechanism by which this crosstalk is achieved molecularly through the mGluR2/2AR complex remains to be elucidated. Two possible molecular mechanisms occurring through cross-conformational changes have been postulated to explain the crosstalk observed between mGluR2 and 2AR through the mGluR2/2AR complex. One

referred to as *cis*-activation, in which the ligand-free receptor is not able to activate G proteins unless the first one signals and is bound to G proteins. The second one, referred to as *trans*-activation, in which the signal from the ligand-bound receptor is transmitted to the neighboring receptor which then signals by activating G proteins.

We hypothesize that SYSMOLE can be applied to differentiate between *cis*- and *trans*-activation in the mGluR2/2AR complex in response to a glutamate antipsychotics.

10.7 Experimental Setup

10.7.1 Measuring G_i and G_q Signaling

The serotonin 2AR is a $G_{q/11}$ -coupled GPCR and its endogenous ligand is the neurotransmitter serotonin, whereas the metabotropic glutamate mGluR2 is a $G_{i/o}$ -coupled, pertussis toxin-sensitive GPCR and its endogenous ligand is the neurotransmitter glutamate (for the sake of simplicity, only G_q and G_i proteins will be mentioned henceforth).

In order to dynamically study the signaling through the complex formed by mGluR2 and 2AR we will need an assay that simultaneously distinguishes between G_i and G_q activation, while allowing us to quantify levels of signaling. We decided to use ion channels as reporters of G protein signaling activity. We expressed each of these GPCRs alone or together, but always with ion channels that acted as reporters of GPCR-mediated signaling, in *Xenopus* oocytes, a heterologous system widely used for functional expression of recombinant ion channels (79). We used GIRK4*(or Kir3.4*) to monitor both G_i and G_q activity (Fig. 10.3) (116). GIRK4* is an inwardly rectifying potassium (Kir) channel, whose current is inhibited upon activation of a G_q -coupled receptor and subsequent phospholipase C-mediated PIP_2 (phosphatidylinositol 4,5 bisphosphate) hydrolysis in the plasma membrane. The extent of current inhibition can be used as a measure of G_q activity. GIRK4* activity is also stimulated by the $G_i\beta\gamma$ subunits liberated upon activation of a G_i -coupled receptor (77). Monitoring the extent of GIRK4* current activation in *real-time* (every second) can be used as a measure of G_i and G_q activity.

Currents through the channels were measured in a Two-Electrode Voltage Clamp (TEVC) configuration (see *Appendix A*). A reversal potential for

Figure 10.3: Measuring G_i and G_q signaling in *Xenopus* oocytes. (A) Representative barium-sensitive traces of GIRK4* currents obtained in response to $1\ \mu\text{M}$ serotonin (5-HT) in oocytes expressing 2AR alone, mGluR2 and 2AR together, or mGluR2 alone. Barium (Ba) inhibited GIRK4* currents and allowed to isolate GIRK4* currents. For illustrative purposes traces with the same basal current were chosen. [cartoon] Stimulation of a G_q -coupled receptor such as 2AR, activates phospholipase C (PLC) to hydrolyze plasma membrane phosphatidylinositol 4,5 bisphosphate (PIP_2) resulting in inhibition of the GIRK4* channel current. RGS2 is shown to inhibit G_q signaling. (B) Representative barium-sensitive traces of GIRK4* currents obtained in response to $1\ \mu\text{M}$ glutamate (Glu) in oocytes expressing mGluR2 alone, mGluR2 and 2AR together, or 2AR alone, with Ba control. [cartoon] Stimulation of a G_i -coupled receptor such as mGluR2 activates G proteins that stimulate GIRK4* currents via the $G\beta\gamma$ subunits. Pertussis toxin (PTX) is shown to inhibit G_i signaling.

SNR(dB)	Config.	σ_1	σ_2	σ_3	γ
50.4 ± 3.81	p(100%)	0.30 ± 0.04	$1.73 \times 10^{-3} \pm 4.96 \times 10^{-4}$	$2.73 \times 10^{-3} \pm 1.21 \times 10^{-3}$	-2.29 ± 0.73

Table 10.2: Kinetic Parameters mGluR2/2AR of complex traces. N= 5 traces. p(100%) indicates parallel configuration was obtained for 100% of the traces.

potassium around 0 mV (symmetrical potassium) was achieved by perfusing a high-potassium solution, and the inward current measured by clamping the voltage in the oocyte to -80 mV. For each trace the current was allowed to stabilize and was recorded at this stable level for 30 seconds before serotonin or glutamate was perfused at 1 μ M concentration. We continued recording until the current reached a new stable state. It should be noted the slower dynamics of G_i activation vs. G_q inactivation (Fig. 10.3). Finally, barium, a selective blocker for inwardly-rectifying potassium channels was perfused at 3 mM concentration, in order to eliminate the contribution of other channels in the oocyte (such as leak) and isolate the current of interest, although it was removed from the trace before analysis by SYSMOLE.

10.8 Results

We applied SYSMOLE to derive the molecular kinetic scheme from macroscopic traces obtained from mGluR2/2AR in response to LY379268, a ligand belonging to the promising new class of glutamate antipsychotics (113), which has been shown to elicit a strong crosstalk between the mGluR2 and the 2AR (91, 115). While glutamate and serotonin, the two endogenous neurotransmitters, activate G_i and G_q pathways respectively through the mGluR2/2AR complex, LY379268 can activate both G_q and G_i despite only binding mGluR2 (Fig. 10.4). The input signal for SYSMOLE was a step in concentration of LY379268, and the output signal the macroscopic current through the GIRK4* channel. Since G_i signaling increases current through the channel (downward direction) and G_q signaling decreases the current through the channel (upward direction, Figure 10.3) the traces obtained (Fig. 10.5) were perfectly suited to apply SYSMOLE to understand the relationship between G_i and G_q signaling and derive a molecular kinetic scheme. It should also be noted that the noise level in these traces results in a SNR clearly below the cut-off for error-free detection (see *Chapter 7* and Table 10.2).

Given that both the opening and closing of the reporter channel are faster processes (milliseconds) compared to G_i and G_q activation (seconds) the trace does not capture them, and the resulting molecular kinetic scheme allows us to distinguish states resulting from combinations in which the G_i is OFF (no change in current) or ON (current through the channel is increasing), G_q is OFF (no change in current) or ON (current through the channel is decreasing), or combinations. Application of SYSMOLE to different macroscopic traces obtained in response to LY379268 (Table 10.2) yielded the molecular kinetic scheme associated with the parallel subtraction configuration (Fig. 10.5) with four states, which can be related to different signaling states depending on whether the G_i or G_q signaling is ON or OFF in the complex. Additional controls were performed with injection of only one receptor (mGluR2 or 2AR) and endogenous ligands to ensure that the traces captured the crosstalk effects through mGluR2/5-HT2AR.

From the molecular kinetic scheme, it can be inferred that G_i and G_q signaling are processes that occur in parallel upon LY379268 binding to mGluR2, and that activation of G_q does not require G_i activation. A close look at the fractional occupancy in each state as a function of time (Fig. 10.6) reveals that due to the strong G_i signal activation of mGluR2 by LY379268 (dominant agonist (115)), most of the complex molecules move from the G_i OFF/ G_q OFF state to G_i ON/ G_q OFF state and then transition directly to the G_i ON/ G_q ON state. Interestingly, there is also a fraction of the molecules that transition from the original G_i OFF / G_q OFF state directly to G_i OFF/ G_q ON state, a molecular state that had not been postulated before. This fraction, albeit small, amounts to approximately one third of the total fractional occupancy in the G_i ON/ G_q ON state when the system reaches steady state. Our results support the hypothesis of a *trans*-activation as the mechanism of crosstalk of the mGluR2/2AR heteromeric complex. It should be noted that the assignment of these ON and OFF states does not come directly from SYSMOLE, which is agnostic to the biological interpretation and is here used to generate hypotheses. The molecular existence of these states would need to be explicitly tested in further experiments.

Summary and Conclusions

- SYSMOLE performed strongly to study GPCR heteromeric signaling.

Figure 10.4: *Cis*-signaling vs. *trans*-signaling. (**Top**) Scheme of crosstalk signaling through the mGluR2/2AR complex. In response to the endogenous ligands glutamate and serotonin, the mGluR2/2AR complex signals through G_i and G_q signaling pathways respectively. Certain ligands, such as LY379268, a dominant mGluR2 agonist with antipsychotic properties are able to crosstalk through the complex and activate both G_i and G_q . (**Bottom**) Molecular kinetic schemes associated with the *cis*-activation and the *trans*-activation theories of crosstalk.

Figure 10.5: Representative G-protein sensitive ion-channel (GIRK4*) current trace (output) elicited by a step in LY379268 concentration from 0 to 1 μM (input) (**left**). Molecular kinetic scheme obtained by applying SYSMOLE to the traces (**right**).

Figure 10.6: Fractional occupancies as a function of time of the four states in the molecular kinetic scheme: G_i OFF/G_q OFF, G_i ON/G_q OFF, G_i ON/G_q ON, and G_i OFF/G_q ON. The molecular kinetic scheme postulates a new state G_i OFF/G_q ON and supports the *trans*-activation theory for crosstalk through the complex. Simulations were generated with the mean value of the kinetic parameters ($N = 5$ traces). Kinetic parameters can be found on Table 10.2.

- The molecular kinetic scheme postulates a new state G_i OFF/ G_q ON and supports the *trans*-activation theory for crosstalk through the mGluR2/2AR complex associated with the mechanism of action of glutamate antipsychotics.
- Our results exemplify the usefulness of the SYSMOLE system-identification analytical tool to generate new biologically-relevant hypotheses.

Chapter 11

Conclusions and Future Work

11.1 Summary

A correct characterization of the dynamics of different molecular processes in the cell is fundamental for understanding normal physiological and pathophysiological responses. In most biological systems the direct study of these microscopic molecular processes is not yet possible due to experimental limitations, and only overall average cell responses to stimuli (macroscopic traces) can be obtained. Here we introduced a method that combines computational and analytical approaches to extract information from the macroscopic trace regarding the molecular microscopic processes and the way in which they are combined. This method, SYSMOLE, conveniently describes the dynamics of these microscopic processes in the form of a molecular kinetic scheme analogous to those used in biophysics and pharmacology.

SYSMOLE is implemented in modular fashion. We first detailed the mathematical underpinnings of each of the three modules that are included in SYSMOLE: the Identifier Module, the Classifier Module, and the Molecular Kinetic Converter Module. Through rigorous and systematic evaluations, we explored SYSMOLE's limitations and robustness to noise. We validated the performance of SYSMOLE on experimental traces by correctly identifying the molecular kinetic scheme describing the activation and inactivation of L-type calcium channels, as well as the mechanism of action of nifedipine, a calcium channel blocker clinically used in patients with cardiovascular disease. Furthermore, we applied SYSMOLE to study the signaling crosstalk mechanism observed in the heteromeric complex formed by mGluR2 and 2AR in response to LY379268, a compound that belongs to the new class of glutamate antipsychotics (92). A Matlab toolbox with

all the functions that we have developed is available in Matlab Central (<http://www.mathworks.com/matlabcentral/61465-sysmole>). The SYSMOLE toolbox contains an implementation of the modules for second-order systems and third-order systems with three poles and two zeros. It also includes the experimental data used in the manuscript, and provides the user with templates to simulate and test noise models and apply SYSMOLE to traces from their biological system of interest.

11.2 Limitations

The overall performance of SYSMOLE is determined by the module with the lowest performance, which in turn depends on the characteristics of each particular macroscopic trace and physiological process under study. The performance of the ARX (autoregressive) method used in the Identifier Module will degrade as the noise increases, or as the sampling frequency decreases and the input and output traces become insufficiently sampled in time. Based on our experience these limitations can be overcome through the application of classical preprocessing and filtering techniques to the traces (46) (see *Chapter 7*) together with an acquisition sampling frequency of $1/2T$, with T being the time constant of the fastest process that we wish to capture. The Classifier Module will not perform properly if no general information regarding the differences in time constants of the processes under study is available. Lacking this information will result in an improperly bound optimization problem which will not exclude the parameter region where the feedback and parallel solutions coincide (see *Chapter 5*). This might hinder the applicability of the method to processes with extremely similar time constants. Finally, the Molecular Kinetic Converter is an analytical conversion, and thus in principle will always perform correctly. To confirm the validity of the conversion for every case, internal checks are included along the mathematical derivation to ensure the rules presented are sound (see *Chapter 6*).

For didactic simplicity, we focused in our presentation on a second-order system, in which two first-order processes are combined in different arrangements. However, each module in SYSMOLE can be scaled to multiple processes. The ARX implementation of the Identifier module can easily extract up to 6 to 10 processes and does not represent a bottleneck for scalability (48, 66). The series of comparisons implemented in the Classifier Module can be updated to incorporate additional combinations of numbers of poles and zeros. The optimization problems needed to differentiate these additional combinations are also easily scalable to higher-order systems, and thus to

multiple processes. Finally, the Molecular Kinetic Scheme can be scaled using a modular approach in which each combination of two processes is considered as a module, and the connection of that module to another module or process also occurs through three possible canonical configurations (see *Chapter 8* for a description of the application of SYSMOLE to third-order systems with three poles and two zeros).

11.3 Comparison with Other Methods

Compared to other methods (see *Chapter 2 section 2.3 Current Methods*) that deal with adequately-sampled data, SYSMOLE is a flexible method that can be generalized to the study of multiple biological systems and applied to different types of biological traces.

SYSMOLE is best adapted to capture subtle features in adequately-sampled macroscopic traces that underlie essential characteristics of the biological system. As such, SYSMOLE is a complementary approach to other methods, and as the experimental techniques evolve to be able to sample more frequently and acquire more data per unit time, tools like SYSMOLE will become particularly useful to have in the computational biologists' toolbox.

SYSMOLE does not rely on underlying models, it extracts information per input-output trace pair, and it incorporates information about the dynamics of the stimulus. Utilizing a system identification step based on a transfer function allows SYSMOLE to capture the dynamics without relying on a physical model. By extending the concept of a Markov chain and linking one of the states to the input, SYSMOLE can extract the molecular kinetic scheme from macroscopic traces elicited by inputs with various dynamics. Finally, the flexible properties of SYSMOLE have the added benefit to retrospectively allow leveraging of important biological information from the vast repository of macroscopic traces collected through the years by different labs.

11.4 Future Work

The future work can be grouped in two categories: i) expansion of SYSMOLE's capabilities, and ii) application of SYSMOLE to different biological systems.

- The future efforts on the expansion of SYSMOLE’s capabilities include the **implementation for fourth-order systems** guided by the rules described in *Chapter 8*. Beyond fourth-order systems other methods would probably be better adapted to solve the problem of deriving information from macroscopic traces, as the interpretation of molecular kinetic schemes in biological terms will be challenging. Furthermore we would like to extensively test the performance of SYSMOLE for macroscopic traces, other than electrophysiological, whose **sampling is unhomogeneous**, as these types of experimental traces are also fairly common.
- We will explore the application of SYSMOLE to study different biological measurements, such as those obtained from single-cell genomics and epigenomics experiments. We realize studying these will require underlying models similar to those described in *Chapter 7, section 7.4.2*. In the advent of single-cell RNA-seq and single-cell ATAC-seq experimental techniques that allow us to measure transcriptomics and epigenomics in single cells (117–119), a tool like SYSMOLE could prove invaluable to gain insight on the mechanisms governing cell transitions between states.

Appendix A

Materials and methods

A.1 Drugs

5-hydroxytryptamine (serotonin, 5-HT), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), acetylcholine (ACh), and (+)-MK801 hydrogen maleate (MK801) were purchased from Sigma-Aldrich. (1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268; LY37), were obtained from Tocris Cookson Inc.

A.2 Molecular Constructs

2AR and mGluR2 constructs were digested with BamHI and NotI and subcloned from pCDNA3.1 into the pXOOM vector. The active point mutants of the human Kir3.4, Kir3.4-S143T or Kir3.4*, constructs had previously been subcloned in pGEMHE, a *Xenopus* oocyte expression vector.

A.3 Expression of Recombinant Proteins in *Xenopus* oocytes

Expression of proteins in oocytes was accomplished by injection of the desired amount of mRNA into *Xenopus oocytes*. In all two-electrode voltage-clamp experiments (TEVC) oocytes were injected with 1 ng of mGluR2, 2 ng of 2AR, and 2 ng of GIRK4* mRNA. Oocytes were isolated and microinjected with equal volumes (50 nl), as previously described (120). All oocytes were maintained at 18°C and recordings were performed 1-4 days following injection adjusting for differences in translation kinetics of each construct.

A.4 Two-Electrode Voltage-Clamp Recording and Analysis

Whole-cell currents were measured by conventional two-microelectrode voltage clamp (TEVC) with a GeneClamp 500 amplifier (Axon Instruments, Union City, CA), as previously reported. A high-potassium (HK) solution was used to superfuse oocytes (96 mM KCl, 1 mM NaCl, 1 mM MgCl₂, 5 mM KOH/HEPES; pH 7.4) to obtain a reversal potential for potassium (EK) close to zero.

Inwardly rectifying potassium currents through GIRK4* were obtained by clamping the cells at -80 mV. Basal GIRK4* currents were defined as the difference between inward currents obtained at -80 mV in the presence of 3 mM BaCl₂ in HK solution and those in the absence of Ba²⁺ and measured for each trace. Current inhibition and current activation were measured respectively and normalized to basal current to compensate for size variability in oocytes.

Bibliography

- [1] Muller-Decker K Marks F, Klingmuller U. *Cellular signal processing. An introduction to the molecular mechanisms of signal transduction*. Garland Science. Taylor and Francis Group, LLC, Upper Saddle River, first edition, 2009.
- [2] D. Bray. Protein molecules as computational elements in living cells. *Nature*, 376(6538):307–12, 1995.
- [3] A. L. Barabasi and Z. N. Oltvai. Network biology: understanding the cell’s functional organization. *Nat Rev Genet*, 5(2):101–13, 2004.
- [4] E. Neher and B. Sakmann. The patch clamp technique. *Sci Am*, 266(3):44–51, 1992.
- [5] O. P. Hamill, A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch*, 391(2):85–100, 1981.
- [6] F. Qin, A. Auerbach, and F. Sachs. Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys J*, 70(1):264–80, 1996.
- [7] F. Qin, A. Auerbach, and F. Sachs. A direct optimization approach to hidden Markov modeling for single channel kinetics. *Biophys J*, 79(4):1915–27, 2000.
- [8] L. H. Hartwell, J. J. Hopfield, S. Leibler, and A. W. Murray. From molecular to modular cell biology. *Nature*, 402(6761 Suppl):C47–52, 1999.
- [9] J. D. Jordan, E. M. Landau, and R. Iyengar. Signaling networks: the origins of cellular multitasking. *Cell*, 103(2):193–200, 2000.

- [10] L. S. Liebovitch and J. Fischbarg. Determining the kinetics of membrane pores from patch clamp data without measuring the open and closed times. *Biochim Biophys Acta*, 813(1):132–6, 1985.
- [11] R. Horn. Statistical methods for model discrimination. Applications to gating kinetics and permeation of the acetylcholine receptor channel. *Biophys J*, 51(2):255–63, 1987.
- [12] Bertil Hille. *Ionic channels of excitable membranes*. Sinauer Associates: Sunderland, MA, 3rd edition, 2001.
- [13] Harris T. E. On chains of infinite order. *Pac J. Math.*, (5):707–724, 1955.
- [14] J.G. Kemeny and Snell J.L. *Finite Markov chains*. Van Nostrand, Princeton, US, 1960.
- [15] Simon H.A. and Ando A. Aggregation of variables in dynamic systems. *Econometrica*, (29):11–138, 1961.
- [16] Fritchman B. D. A binary channel characterization using partitioned Markov chains. *IEEE Information Theory*, 13:221–227, 1967.
- [17] D. R. Montal M Fredkin and Rkice J. A. Identification of aggregated Markovian models; application to the nicotinic acetylcholine receptor. *Proc. Berkeley Conf. in Honor of J. Neyman and J. Kiefer*, 1(Belmon, California: Wadsworth):269–289, 1985.
- [18] Rabiner L.R. and Juang B.H. An introduction to hidden Markov models. *ASSP Magazine*, (3):14–16, 1986.
- [19] Blackwell D. and Koopmans L. On the identifiability problem for functions of finite Markov chains. *Ann. Math. Stat.*, (28):1011–1015, 1957.
- [20] Gilbert E.J. On the identifiability problem for functions of finite Markov chains. *Ann. Math. Stat.*, (30):688–687, 1982.
- [21] Petrie T. Probabilistic functions of finite state Markov chains. *Ann. Math. Stat.*, (40):97–115, 1969.
- [22] Erickson R.V. Functions of Markov chains. *Ann. Math. Stat.*, (41):843–850, 1970.
- [23] Greeno J.G. Wandell B.A. and 1974 Egan D.E. Equivalence classes of functions of finite Markov chains. *J. math. Psych.*, (11):391–403, 1974.

- [24] P. Kienker. Equivalence of aggregated Markov models of ion-channel gating. *Proc R Soc Lond B Biol Sci*, 236(1284):269–309, 1989.
- [25] C. Schnbach. From immunogenetics to immunomics: functional prospecting of genes and transcripts. *Novartis Found Symp*, 254:177–88; discussion 189–92, 216–22, 250–2, 2003.
- [26] L. D. Mcclenny, M. Imani, and U. M. Braga-Neto. Boolfilter: an R package for estimation and identification of partially-observed boolean dynamical systems. *BMC Bioinformatics*, 18(1):519, 2017.
- [27] U. M. Braga-Neto and E. T. Marques. From functional genomics to functional immunomics: new challenges, old problems, big rewards. *PLoS Comput Biol*, 2(7):e81, 2006.
- [28] T. Chen and U. M. Braga-Neto. Statistical detection of boolean regulatory relationships. *IEEE/ACM Trans Comput Biol Bioinform*, 10(5):1310–21, 2013.
- [29] T. Thorne and M. P. Stumpf. Inference of temporally varying Bayesian networks. *Bioinformatics*, 28(24):3298–305, 2012.
- [30] T. Thorne and M. P. Stumpf. Graph spectral analysis of protein interaction network evolution. *J R Soc Interface*, 9(75):2653–66, 2012.
- [31] D. K. Ralph and F. A. Matsen. Consistency of VDJ rearrangement and substitution parameters enables accurate B cell receptor sequence annotation. *PLoS Comput Biol*, 12(1):e1004409, 2016.
- [32] A. C. Babbie, P. Kirk, and M. P. Stumpf. Topological sensitivity analysis for systems biology. *Proc Natl Acad Sci U S A*, 111(52):18507–12, 2014.
- [33] D. M. Gysi, A. Voigt, T. M. Fragoso, E. Almaas, and K. Nowick. wTO: an R package for computing weighted topological overlap and a consensus network with integrated visualization tool. *BMC Bioinformatics*, 19(1):392, 2018.
- [34] A. Taherkhani, S. Kalantari, A. Arefi Oskouie, M. Nafar, M. Taghizadeh, and K. Tabar. Network analysis of membranous glomerulonephritis based on metabolomics data. *Mol Med Rep*, 18(5):4197–4212, 2018.

- [35] C. J. Oates, F. Dondelinger, N. Bayani, J. Korkola, J. W. Gray, and S. Mukherjee. Causal network inference using biochemical kinetics. *Bioinformatics*, 30(17):i468–74, 2014.
- [36] S. Barbosa, B. Niebel, S. Wolf, K. Mauch, and R. Takors. A guide to gene regulatory network inference for obtaining predictive solutions: Underlying assumptions and fundamental biological and data constraints. *Biosystems*, 174:37–48, 2018.
- [37] D. J. Warne, R. E. Baker, and M. J. Simpson. Simulation and inference algorithms for stochastic biochemical reaction networks: from basic concepts to state-of-the-art. *J R Soc Interface*, 16(151):20180943, 2019.
- [38] J. J. Celentano and A. G. Hawkes. Use of the covariance matrix in directly fitting kinetic parameters: application to GABAA receptors. *Biophys J*, 87(1):276–94, 2004.
- [39] F. Marni, S. Wu, G. M. Shah, X. P. Xu, A. R. Hackett, C. Xie, S. Shrestha, L. Liu, Q. Liu, and L. Zhou. Normal-mode-analysis-guided investigation of crucial intersubunit contacts in the cAMP-dependent gating in HCN channels. *Biophys J*, 103(1):19–28, 2012.
- [40] L. S. Milescu, G. Akk, and F. Sachs. Maximum likelihood estimation of ion channel kinetics from macroscopic currents. *Biophys J*, 88(4):2494–515, 2005.
- [41] L. S. Milescu, T. Yamanishi, K. Ptak, M. Z. Mogri, and J. C. Smith. Real-time kinetic modeling of voltage-gated ion channels using dynamic clamp. *Biophys J*, 95(1):66–87, 2008.
- [42] A. Salari, M. A. Navarro, M. Milescu, and L. S. Milescu. Estimating kinetic mechanisms with prior knowledge I: Linear parameter constraints. *J Gen Physiol*, 150(2):323–338, 2018.
- [43] M. A. Navarro, A. Salari, M. Milescu, and L. S. Milescu. Estimating kinetic mechanisms with prior knowledge II: Behavioral constraints and numerical tests. *J Gen Physiol*, 150(2):339–354, 2018.
- [44] Mihai Glont, Tung V. N. Nguyen, Martin Graesslin, Robert Hlke, Raza Ali, Jochen Schramm, Sarala M. Wimalaratne, Varun B. Kothamachu, Nicolas Rodriguez, Maciej J. Swat, Jurgen Eils, Roland Eils, Camille Laibe, Rahuman S. Malik-Sheriff, Vijayalakshmi Chelliah, Nicolas Le Novre, and Henning Hermjakob. BioModels: expanding horizons

- to include more modelling approaches and formats. *Nucleic Acids Research*, 46(D1):D1248D1253, 2018.
- [45] Hamid-Nawab S. Oppenheim AV, Willsky AS. *Signals and systems*. Prentice Hall, Upper Saddle River, NJ, 2nd edition, 1999.
- [46] Godfrey K R. *Perturbation Signals for System Identification*. Prentice Hall, London, UK, 1993.
- [47] S. Nobukawa and N. Shibata. Controlling chaotic resonance using external feedback signals in neural systems. *Sci Rep*, 9(1):4990, 2019.
- [48] Lennart Ljung. *System Identification: Theory for the User*. Information and System Sciences Series. Prentice Hall PTR, Upper Saddle River, second edition, 1999.
- [49] C Mei. On teaching simplification of block diagrams. *Int. Journal of Engng Ed*, 18:697–703, 2002.
- [50] NM Karayanakis. *Advanced system modeling and simulation with block diagram languages*. CRC Press, Boca Raton, Florida, US, 1995.
- [51] J Dennis and L Vicente. Trust-region interior-point algorithms for minimization problems with simple bounds. *Applied Mathematics and Parallel Computing*, 1996.
- [52] S. Hoops, S. Sahle, R. Gauges, C. Lee, J. Pahle, N. Simus, M. Singhal, L. Xu, P. Mendes, and U. Kummer. COPASI—a COMplex PATHway SIMulator. *Bioinformatics*, 22(24):3067–74, 2006.
- [53] D. Colquhoun and A. G. Hawkes. On the stochastic properties of single ion channels. *Proc R Soc Lond B Biol Sci*, 211(1183):205–35, 1981.
- [54] L. Song and K. L. Magleby. Testing for microscopic reversibility in the gating of maxi K⁺ channels using two-dimensional dwell-time distributions. *Biophys J*, 67(1):91–104, 1994.
- [55] B. S. Rothberg and K. L. Magleby. Testing for detailed balance (microscopic reversibility in ion channel gating. *Biophys J*, 80(6):3025–6, 2001.
- [56] J. Patlak. Molecular kinetics of voltage-dependent na⁺ channels. *Physiol Rev*, 71(4):1047–80, 1991.

- [57] O. P. Hamill, A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch*, 391(2):85–100, 1981.
- [58] D. Colquhoun and B. Sakmann. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J Physiol*, 369:501–57, 1985.
- [59] R. D. Astumian. Thermodynamics and kinetics of a brownian motor. *Science*, 276(5314):917–22, 1997.
- [60] R. D. Astumian and I. Deryni. Fluctuation driven transport and models of molecular motors and pumps. *Eur Biophys J*, 27(5):474–89, 1998.
- [61] M. B. Elowitz, A. J. Levine, E. D. Siggia, and P. S. Swain. Stochastic gene expression in a single cell. *Science*, 297(5584):1183–6, 2002.
- [62] W. J. Blake, M. KAern, C. R. Cantor, and J. J. Collins. Noise in eukaryotic gene expression. *Nature*, 422(6932):633–7, 2003.
- [63] N. Maheshri and E. K. O’Shea. Living with noisy genes: how cells function reliably with inherent variability in gene expression. *Annu Rev Biophys Biomol Struct*, 36:413–34, 2007.
- [64] S. Patil, M. Fribourg, Y. Ge, M. Batish, S. Tyagi, F. Hayot, and S. C. Sealfon. Single-cell analysis shows that paracrine signaling by first responder cells shapes the interferon- response to viral infection. *Sci Signal*, 8(363):ra16, 2015.
- [65] M. Fribourg, B. Hartmann, M. Schmolke, N. Marjanovic, R. A. Albrecht, A. Garca-Sastre, S. C. Sealfon, C. Jayaprakash, and F. Hayot. Model of influenza a virus infection: dynamics of viral antagonism and innate immune response. *J Theor Biol*, 351:47–57, 2014.
- [66] Sderstrm T, Fan H, Carlsson B, and Bigi S. Least squares parameter estimation of continuous-time ARX models from discrete-time data. *IEEE Transaction on Automatic Control*, 42(5), 1997.
- [67] Jessell T M Kandel E R, Schwartz J H. *Principles of neural science*. McGraw Hill, fourth edition, 2000.
- [68] B. E. Flucher. Specific contributions of the four voltage-sensing domains in l-type calcium channels to gating and modulation. *J Gen Physiol*, 148(2):91–5, 2016.

- [69] B. P. Bean. Classes of calcium channels in vertebrate cells. *Annu Rev Physiol*, 51:367–84, 1989.
- [70] E. Carbone and D. Swandulla. Neuronal calcium channels: kinetics, blockade and modulation. *Prog Biophys Mol Biol*, 54(1):31–58, 1989.
- [71] R. H. Scott, H. A. Pearson, and A. C. Dolphin. Aspects of vertebrate neuronal voltage-activated calcium currents and their regulation. *Prog Neurobiol*, 36(6):485–520, 1991.
- [72] R. W. Tsien, D. Lipscombe, D. V. Madison, K. R. Bley, and A. P. Fox. Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci*, 11(10):431–8, 1988.
- [73] A. S. Go, M. A. Bauman, S. M. Coleman King, G. C. Fonarow, W. Lawrence, K. A. Williams, E. Sanchez, American Heart Association, American College of Cardiology, and Centers for Disease Control and Prevention. An effective approach to high blood pressure control: a science advisory from the American Heart Association, the American College of Cardiology, and the Centers for Disease Control and Prevention. *Hypertension*, 63(4):878–85, 2014.
- [74] C. W. Yancy, M. Jessup, B. Bozkurt, J. Butler, D. E. Casey, M. H. Drazner, G. C. Fonarow, S. A. Geraci, T. Horwich, J. L. Januzzi, M. R. Johnson, E. K. Kasper, W. C. Levy, F. A. Masoudi, P. E. McBride, J. J. McMurray, J. E. Mitchell, P. N. Peterson, B. Riegel, F. Sam, L. W. Stevenson, W. H. Tang, E. J. Tsai, and B. L. Wilkoff. 2013 ACCF/AHA guideline for the management of heart failure: executive summary: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*, 128(16):1810–52, 2013.
- [75] V. Brezina, C. G. Evans, and K. R. Weiss. Characterization of the membrane ion currents of a model molluscan muscle, the accessory radula closer muscle of *Aplysia californica*. III. depolarization-activated Ca current. *J Neurophysiol*, 71(6):2126–38, 1994.
- [76] A. M. Gurney, J. M. Nerbonne, and H. A. Lester. Photoinduced removal of nifedipine reveals mechanisms of calcium antagonist action on single heart cells. *J Gen Physiol*, 86(3):353–79, 1985.
- [77] D. E. Logothetis, Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. The beta gamma subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature*, 325(6102):321–6, 1987.

- [78] J. Gonzalez-Maeso, N. V. Weisstaub, M. Zhou, P. Chan, L. Ivic, R. Ang, A. Lira, M. Bradley-Moore, Y. Ge, Q. Zhou, S. C. Sealton, and J. A. Gingrich. Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron*, 53(3):439–52, 2007.
- [79] E. Kobrinisky, T. Mirshahi, H. Zhang, T. Jin, and D. E. Logothetis. Receptor-mediated hydrolysis of plasma membrane messenger PIP2 leads to K⁺-current desensitization. *Nat Cell Biol*, 2(8):507–14, 2000.
- [80] K. Sriram and P. A. Insel. G Protein-coupled receptors as targets for approved drugs: How many targets and how many drugs? *Mol Pharmacol*, 93(4):251–258, 2018.
- [81] M. R. Whorton, M. P. Bokoch, S. G. Rasmussen, B. Huang, R. N. Zare, B. Kobilka, and R. K. Sunahara. A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci U S A*, 104(18):7682–7, 2007.
- [82] M. R. Whorton, B. Jastrzebska, P. S. Park, D. Fotiadis, A. Engel, K. Palczewski, and R. K. Sunahara. Efficient coupling of transducin to monomeric rhodopsin in a phospholipid bilayer. *J Biol Chem*, 283(7):4387–94, 2008.
- [83] A. J. Kuszak, S. Pitchaiya, J. P. Anand, H. I. Mosberg, N. G. Walter, and R. K. Sunahara. Purification and functional reconstitution of monomeric mu-opioid receptors: allosteric modulation of agonist binding by Gi2. *J Biol Chem*, 284(39):26732–41, 2009.
- [84] O. P. Ernst, V. Gramse, M. Kolbe, K. P. Hofmann, and M. Heck. Monomeric G protein-coupled receptor rhodopsin in solution activates its G protein transducin at the diffusion limit. *Proc Natl Acad Sci U S A*, 104(26):10859–64, 2007.
- [85] S. Ferr, R. Baler, M. Bouvier, M. G. Caron, L. A. Devi, T. Durroux, K. Fuxe, S. R. George, J. A. Javitch, M. J. Lohse, K. Mackie, G. Milligan, K. D. Pfleger, J. P. Pin, N. D. Volkow, M. Waldhoer, A. S. Woods, and R. Franco. Building a new conceptual framework for receptor heteromers. *Nat Chem Biol*, 5(3):131–4, 2009.
- [86] W. Z. Zhu, K. Chakir, S. Zhang, D. Yang, C. Lavoie, M. Bouvier, T. E. Hbert, E. G. Lakatta, H. Cheng, and R. P. Xiao. Heterodimerization of beta1- and beta2-adrenergic receptor subtypes optimizes beta-

- adrenergic modulation of cardiac contractility. *Circ Res*, 97(3):244–51, 2005.
- [87] L. Barki-Harrington, L. M. Luttrell, and H. A. Rockman. Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: a functional role for receptor-receptor interaction in vivo. *Circulation*, 108(13):1611–8, 2003.
- [88] S. AbdAlla, A. Abdel-Baset, H. Lothar, A. el Massiery, and U. Quitterer. Mesangial AT1/B2 receptor heterodimers contribute to angiotensin II hyperresponsiveness in experimental hypertension. *J Mol Neurosci*, 26(2-3):185–92, 2005.
- [89] D. W. McGraw, K. A. Mihlbachler, M. R. Schwarb, F. F. Rahman, K. M. Small, K. F. Almoosa, and S. B. Liggett. Airway smooth muscle prostaglandin-EP1 receptors directly modulate beta2-adrenergic receptors within a unique heterodimeric complex. *J Clin Invest*, 116(5):1400–9, 2006.
- [90] H. J. Chun, Z. A. Ali, Y. Kojima, R. K. Kundu, A. Y. Sheikh, R. Agrawal, L. Zheng, N. J. Leeper, N. E. Pearl, A. J. Patterson, J. P. Anderson, P. S. Tsao, M. J. Lenardo, E. A. Ashley, and T. Quertemous. Apelin signaling antagonizes Ang II effects in mouse models of atherosclerosis. *J Clin Invest*, 118(10):3343–54, 2008.
- [91] J. Gonzalez-Maeso, R. L. Ang, T. Yuen, P. Chan, N. V. Weisstaub, J. F. Lopez-Gimnez, M. Zhou, Y. Okawa, L. F. Callado, G. Milligan, J. A. Gingrich, M. Filizola, J. J. Meana, and S. C. Sealton. Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature*, 452(7183):93–7, 2008.
- [92] A. Ellaithy, J. Younkin, J. Gonzalez-Maeso, and D. E. Logothetis. Positive allosteric modulators of metabotropic glutamate 2 receptors in schizophrenia treatment. *Trends Neurosci*, 38(8):506–16, 2015.
- [93] J. L. Moreno, P. Miranda-Azpiazu, A. Garca-Bea, J. Younkin, M. Cui, A. Kozlenkov, A. Ben-Ezra, G. Voloudakis, A. K. Fakira, L. Baki, Y. Ge, A. Georgakopoulos, J. A. Morn, G. Milligan, J. F. Lopez-Gimnez, N. K. Robakis, D. E. Logothetis, J. J. Meana, and J. Gonzalez-Maeso. Allosteric signaling through an mGlu2 and 5-HT2A heteromeric receptor complex and its potential contribution to schizophrenia. *Sci Signal*, 9(410):ra5, 2016.

- [94] A. Sawa and S. H. Snyder. Schizophrenia: diverse approaches to a complex disease. *Science*, 296(5568):692–5, 2002.
- [95] C. A. Ross, R. L. Margolis, S. A. Reading, M. Pletnikov, and J. T. Coyle. Neurobiology of schizophrenia. *Neuron*, 52(1):139–53, 2006.
- [96] H. Y. Meltzer. Suicidality in schizophrenia: a review of the evidence for risk factors and treatment options. *Curr Psychiatry Rep*, 4(4):279–83, 2002.
- [97] J. J. Lin, F. W. Liang, C. Y. Li, and T. H. Lu. Leading causes of death among decedents with mention of schizophrenia on the death certificates in the united states. *Schizophr Res*, 2018.
- [98] L. D. Selemon and P. S. Goldman-Rakic. The reduced neuropil hypothesis: a circuit based model of schizophrenia. *Biol Psychiatry*, 45(1):17–25, 1999.
- [99] M. P. Forrest, E. Parnell, and P. Penzes. Dendritic structural plasticity and neuropsychiatric disease. *Nat Rev Neurosci*, 19(4):215–234, 2018.
- [100] T. Kenakin. Drug efficacy at G protein-coupled receptors. *Annu Rev Pharmacol Toxicol*, 42:349–79, 2002.
- [101] V. J. Aloyo, K. A. Berg, U. Spampinato, W. P. Clarke, and J. A. Harvey. Current status of inverse agonism at serotonin2a (5-HT2A) and 5-HT2C receptors. *Pharmacol Ther*, 121(2):160–73, 2009.
- [102] C. T. Egan, K. Herrick-Davis, and M. Teitler. Creation of a constitutively activated state of the 5-hydroxytryptamine 2a receptor by site-directed mutagenesis: inverse agonist activity of antipsychotic drugs. *J Pharmacol Exp Ther*, 286(1):85–90, 1998.
- [103] D. M. Weiner, E. S. Burstein, N. Nash, G. E. Croston, E. A. Currier, K. E. Vanover, S. C. Harvey, E. Donohue, H. C. Hansen, C. M. Andersson, T. A. Spalding, D. F. Gibson, K. Krebs-Thomson, S. B. Powell, M. A. Geyer, U. Hacksell, and M. R. Brann. 5-hydroxytryptamine2A receptor inverse agonists as antipsychotics. *J Pharmacol Exp Ther*, 299(1):268–76, 2001.
- [104] B. J. Morris, S. M. Cochran, and J. A. Pratt. PCP: from pharmacology to modelling schizophrenia. *Curr Opin Pharmacol*, 5(1):101–6, 2005.

- [105] D. Fiorella, R. A. Rabin, and J. C. Winter. Role of 5-HT_{2A} and 5-HT_{2C} receptors in the stimulus effects of hallucinogenic drugs. II: Reassessment of lsd false positives. *Psychopharmacology (Berl)*, 121(3):357–63, 1995.
- [106] L. L. Wing, G. S. Tapson, and M. A. Geyer. 5HT-2 mediation of acute behavioral effects of hallucinogens in rats. *Psychopharmacology (Berl)*, 100(3):417–25, 1990.
- [107] F. X. Vollenweider, M. F. Vollenweider-Scherpenhuyzen, A. Bbler, H. Vogel, and D. Hell. Psilocybin induces schizophrenia-like psychosis in humans via a serotonin-2 agonist action. *Neuroreport*, 9(17):3897–902, 1998.
- [108] A. F. Lehman, J. A. Lieberman, L. B. Dixon, T. H. McGlashan, A. L. Miller, D. O. Perkins, J. Kreyenbuhl, American Psychiatric Association, and Steering Committee on Practice Guidelines. Practice guideline for the treatment of patients with schizophrenia, second edition. *Am J Psychiatry*, 161(2 Suppl):1–56, 2004.
- [109] L. Helldin, J. M. Kane, U. Karilampi, T. Norlander, and T. Archer. Remission in prognosis of functional outcome: a new dimension in the treatment of patients with psychotic disorders. *Schizophr Res*, 93(1-3):160–8, 2007.
- [110] M. De Hert, R. van Winkel, M. Wampers, J. Kane, J. van Os, and J. Peuskens. Remission criteria for schizophrenia: evaluation in a large naturalistic cohort. *Schizophr Res*, 92(1-3):68–73, 2007.
- [111] E. Jskelinen, P. Juola, N. Hirvonen, J. J. McGrath, S. Saha, M. Isohanni, J. Veijola, and J. Miettunen. A systematic review and meta-analysis of recovery in schizophrenia. *Schizophr Bull*, 39(6):1296–306, 2013.
- [112] J. L. Moreno, S. C. Sealton, and J. Gonzalez-Maeso. Group II metabotropic glutamate receptors and schizophrenia. *Cell Mol Life Sci*, 66(23):3777–85, 2009.
- [113] J. L. Moreno, T. Holloway, L. Albizu, S. C. Sealton, and J. Gonzalez-Maeso. Metabotropic glutamate mglu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT_{2A} receptor agonists. *Neurosci Lett*, 493(3):76–9, 2011.

- [114] B. J. Kinon, L. Zhang, B. A. Millen, O. O. Osuntokun, J. E. Williams, S. Kollack-Walker, K. Jackson, L. Kryzhanovskaya, N. Jarkova, and HBBI Study Group. A multicenter, inpatient, phase 2, double-blind, placebo-controlled dose-ranging study of LY2140023 monohydrate in patients with DSM-IV schizophrenia. *J Clin Psychopharmacol*, 31(3):349–55, 2011.
- [115] M. Fribourg, J. L. Moreno, T. Holloway, D. Provasi, L. Baki, R. Mahajan, G. Park, S. K. Adney, C. Hatcher, J. M. Eltit, J. D. Ruta, L. Albizu, Z. Li, A. Umali, J. Shim, A. Fabiato, A. D. MacKerell, V. Brezina, S. C. Sealton, M. Filizola, J. Gonzalez-Maeso, and D. E. Logothetis. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell*, 147(5):1011–23, 2011.
- [116] C. Hatcher-Solis, M. Fribourg, K. Spyridaki, J. Younkin, A. Ellaithy, G. Xiang, G. Liapakis, J. Gonzalez-Maeso, H. Zhang, M. Cui, and D. E. Logothetis. G protein-coupled receptor signaling to kir channels in xenopus oocytes. *Curr Pharm Biotechnol*, 15(10):987–95, 2014.
- [117] J. G. Camp, D. Wollny, and B. Treutlein. Single-cell genomics to guide human stem cell and tissue engineering. *Nat Methods*, 15(9):661–667, 2018.
- [118] P. K. Lo and Q. Zhou. Emerging techniques in single-cell epigenomics and their applications to cancer research. *J Clin Genom*, 1(1), 2018.
- [119] S. Dasgupta, G. D. Bader, and S. Goyal. Single-cell RNA sequencing: A new window into cell scale dynamics. *Biophys J*, 115(3):429–435, 2018.
- [120] C. M. Lopes, H. Zhang, T. Rohacs, T. Jin, J. Yang, and D. E. Logothetis. Alterations in conserved Kir channel-PIP2 interactions underlie channelopathies. *Neuron*, 34(6):933–44, 2002.