# Analysis of the Signal Transduction Dynamics Regulating mTOR with Mathematical Modeling, Petri Nets and Dynamic Graphs

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**Abstract.** Signaling networks in the mammalian cell are complex systems. Their dynamic properties can often be explained by the interaction of regulatory network motifs. Mathematical modeling is instrumental in explaining how these systems function. To accomplish this task in this paper, we combine numerical simulations of differential equations, which produce the individual trajectories of protein concentrations, and structural analysis of the reaction network with Petri nets. In the end, we generate dynamic graphs to get a systems view of the signaling network dynamics. In this paper, we report initial work on the regulatory network of the protein mTOR. In neuronal synaptic plasticity, prolonged activation of this protein is needed to support an increased protein synthesis. However, biologists wonder how two brief calcium influxes of 1 second each can lead to this long activation downstream. With our computational approach, we explore a simple hypothesis for the response of mTOR, the crosstalk between the Akt and Wnt pathways, with two different models. Initial results suggest that this mechanism alone cannot explain the experimental data.

**Keywords:** Computational biology, Cell signaling, Mathematical modeling, Petri nets, mTOR, Synaptic plasticity

## 1 Introduction

Signaling pathways are the communication system of the cell. They transmit and process information. These pathways form a complex network with several regulatory mechanisms and often exhibit emergent properties. Computational biology approaches have made several theoretical contributions to the analysis and understanding of these molecular and cellular systems using modeling and simulation. Some even led to experimental discoveries.

Synaptic plasticity in neurons is a collection of systems properties that has long been intriguing neurobiologists. Most importantly, it has been shown that

synaptic plasticity is the cellular correlate of learning and memory in the nervous system [19]. One of these properties is long-term potentiation (LTP), a persistent strengthening of synapses following certain patterns of electrical activity. Whether in the early induction phase or the late maintenance phase, LTP relies on the biochemical regulation of different signaling pathways. For example, the activity of the protein mammalian target of rapamycin (mTOR) – a modulator of the translation capacity of the cell – is required during the late phase of LTP (L-LTP) to increase protein synthesis [22]. Interestingly, a recent study linked memory impairments caused by sleep deprivation to a molecular mechanism involving mTOR [23]. If mTOR is inhibited, potentiated synapses revert to their initial state. Ma et al. showed that the activity of two cell signaling pathways, the Akt pathway and the Wnt pathway, is required to activate mTOR and induce L-LTP [15]. Despite experimental data supporting this signaling mechanism, some questions about its dynamics remain unanswered. How is it possible that a strong, but very brief, synaptic stimulation can trigger the prolonged activation of mTOR needed to induce L-LTP? How can two 1-second calcium influxes lead to a 45-minute mTOR response? This kind of question is best answered with the help of theoretical analyses.

In this paper, we present the approach that we will be using to study this question. We plan to eventually explore several signaling mechanisms that might explain this temporal prolongation of the signal initiated by synaptic activity. We will model each mechanism with reaction-based ordinary differential equations (ODE) and perform numerical simulations. We will also make a structural analysis of the reaction network with Petri nets [7]. We will visualize the simulation data in a graph-based representation that our group developed [1, 6]. Our goal is twofold: first study different network motifs that could produce such an effect and gain a better understanding of their dynamic properties with our dynamic graphs; and second identify likely biological mechanisms to latter test experimentally.

In the preliminary work presented here, we focus on a simple interaction between the Akt pathway and the Wnt pathway. Precisely, we model the cross-regulation by Akt of GSK3, a kinase downstream of the Wnt pathway. This creates a feed-forward motif that Ma et al. hypothesized to be responsible for the surprisingly long activation of mTOR [15]. We first combined two existing mathematical models to create an integrated signaling model. To have a better agreement between the experimental and simulation data, we created a more detailed model with additional molecular mechanisms found in the literature. In the following sections, we present the two models, their simulation results and the resulting dynamic graphs. Our initial analysis suggests that this network motif increases the amplitude of the downstream signal and acts as a coincidence detector but that it cannot fully achieve the experimentally observed prolongation of the mTOR activation.

## 2 Two iterations of mathematical modeling of the Akt and Wnt signaling pathways and simulation results

### 2.1 First modeling iteration: a model of a simple Akt-Wnt crosstalk

In neurons, one of the initial events that can trigger signal transduction on many signaling pathways is the entry of calcium ions through channels or neurotransmitter receptors. Calcium is a secondary messenger and once it enters the cell, it activates various proteins. One of the signaling cascades activated by calcium that culminates with mTOR is the Akt pathway. This pathway was modeled by Jain and Bhalla as part of a larger signaling network involving the growth factor BDNF and its effect on the activity of mTOR [11]. Their model also included the protein synthesis process. With this model, Jain and Bhalla studied if a bistable regime was possible. Bistability could arise from a self-sustaining positive feedback loop formed by the synthesis of proteins that are involved in the signaling network controlling protein synthesis itself through mTOR. Jain and Bhalla concluded from their simulation results that the currently known feedback mechanisms do not allow for such a switch.

To assemble our Akt-Wnt-mTOR model (shown in Figure 1), we reused part of the model developed by Jain and Bhalla. Calcium (Ca) activates calmoduline (CaM) and then a guanine nucleotide exchange factor (GEF). GEF in turn activates the GTPase Ras, which then binds to the phosphoinositide 3-kinase (PI3K). Once active, this kinase initiates the production of phosphatidylinositol 3-phosphate (PIP3), a membrane bound phospholipid that recruits the proteins PDK1 and Akt to the membrane where they both become active. Once activated, Akt phosphorylates the TSC2 complex (tuberous sclerosis 1-tuberous sclerosis 2) and GSK3, inhibiting both proteins. The inactivation of TSC2 relieves the repression it exerts on the GTP-binding protein Rheb. This last protein can then bind to mTOR and activate it.

Experimental data also linked the Wnt pathway to the regulation of mTOR in neurons through GSK3 [15]. Wnt is a ligand secreted for autocrine and paracrine signaling. It has been shown that synaptic stimulation causes the exocytosis of Wnt in the extracellular space where the ligand binds to the receptor Frizzled, which passes the signal to the intracellular protein Disheveled (Dsh). The activated Dsh protein then recruits the proteins Axin and GSK3 to the receptor, preventing the formation of the destruction complex by these proteins with APC. When operational, this complex phosphorylates the transcription factor $\beta$-catenin, marking it for degradation. Another target of the destruction complex is TSC2. When GSK3 is in the complex, this protein maintains a basal activation of TSC2, thus tonically repressing mTOR. This repression is therefore removed by Wnt signaling. To add this pathway to our dynamical model, we incorporated the work from Tan et al [21]. This model is an adaptation for mammalian cells of the classical Wnt signaling model by Lee et al [13].

The resulting integrated model of the Akt/Wnt pathways is composed of kinetics-based biochemical reactions like mass action reactions and enzymatic reactions. Reaction rates were defined as ordinary differential equations with

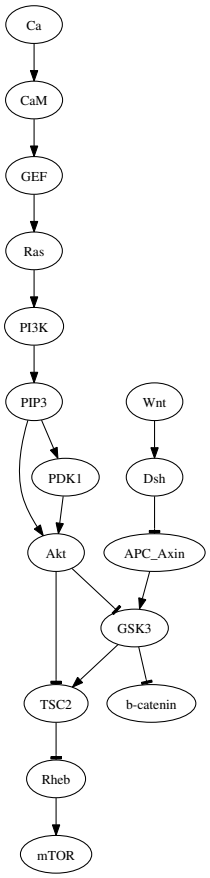


**Fig. 1.** Interaction graph representation of the integrated Akt-Wnt signaling pathways. Regular arrows represent activation relationships and tee arrows, inhibitory relationships. Akt, GSK3 and TSC2 form a feedforward motif.

most parameters and initial conditions coming from the two published papers. Our model contains approximately 50 equations. We implemented the model with the software Virtual Cell [18]. We validated the Wnt part of the model by achieving a $\beta$-catenin time course identical to published simulation results (Figure 2, to be compared with Figure 7A from Tan et al [21]). In this simulation, Wnt is present throughout. As a result, we can see the elevation of the concentration of $\beta$-catenin as its phosphorylation by the destruction complex is reduced, thus reducing the degradation of the transcription factor[1].

[1] The model of the Akt-Wnt-mTOR pathway is freely accessible in the public VCell database under username mapaf2 and model names "mTOR - model 1 (Wnt+GSK3)" and "mTOR - model 2 (Wnt+GSK3)". A file in the SBML format can be exported

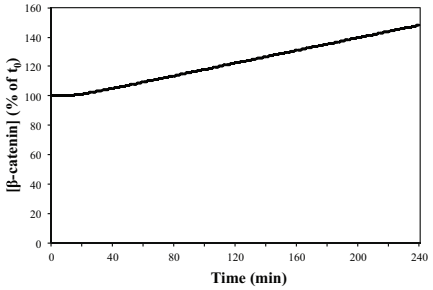


**Fig. 2.** Simulation results for the concentration of $\beta$-catenin when Wnt is continuously present.

Once the model has been set up, the next step was to define a stimulation protocol that replicates a synaptic stimulation known to induce mTOR activity. The experimental protocol used on hippocampal acute slices is high-frequency stimulation (HFS): an electrode delivers two high-frequency trains of stimuli to presynaptic fibers at 100 Hz for 1 second, 20 seconds apart [22]. We defined a calcium influx pattern that results from HFS (see Figure 3, left). Following HFS, it has been shown that Wnt is secreted [4]. Wnt is then found in the extracellular space around the neuron about 10 minutes after HSF and activates the Frizzled receptor for approximately 15 minutes [14]. We defined a Wnt concentration profile that corresponds to these measures (see Figure 3, right).

We simulated the Akt-Wnt-mTOR model with this stimulation protocol and monitored mTOR activity. We also ran a second simulation without the Wnt stimulation to measure the contribution of the Akt pathway alone. The kinetic parameters we used are from the published models except the Michaelis-Menten parameters of the phosphorylation of TSC2 by GSK3. In the first two simulations, we used the same values as the phosphorylation of the other GSK3 substrate $\beta$-catenin. The simulation results are shown in Figure 4, at the left. With the kinetic constants from the published models, the contribution of the

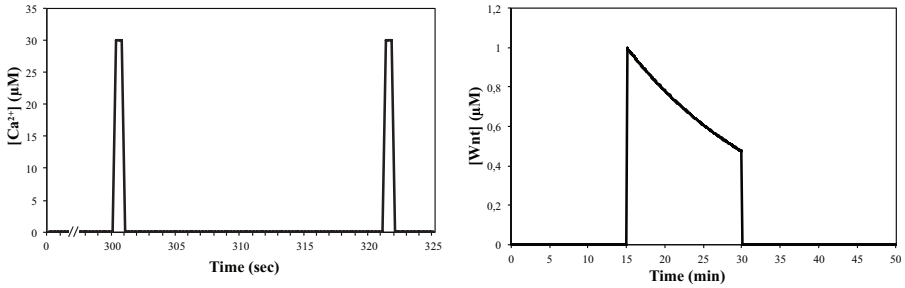


**Fig. 3.** Stimulation patterns for the molecular inputs of the model: calcium (left) and Wnt (right).

Wnt pathway to mTOR activity (solid line) seems negligible compared to the contribution of the Akt pathway alone (dashed line). This simulation result is in contradiction with the published experimental data from Ma et al where inhibition of either the Wnt pathway or the Akt pathway blocks the activation of mTOR [15].

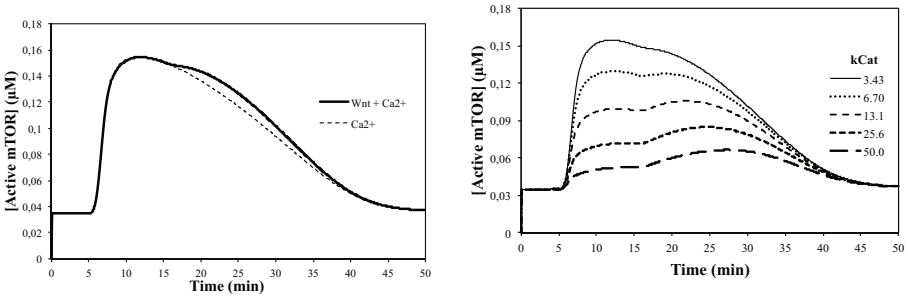


**Fig. 4.** Simulated activation of mTOR in the first iteration model. On the left, simulations with both calcium and Wnt stimulations and with calcium alone. On the right, simulations with both calcium and Wnt stimulations for various kcat values of the TSC2 phosphorylation by GSK3.

Since the kinetic parameters of the TSC2-GSK3 phosphorylation were estimated, we explored other values from the original value 3 $s^{-1}$ up to 50 $s^{-1}$. Simulation results are shown in Figure 4, at the right. Increasing this kcat globally lowered mTOR activity, which was expected since GSK3 becomes a more efficient activator of TSC2 with a higher kcat. Another effect was a bigger contribution of the Wnt pathway to mTOR activation in comparison to the Akt pathway. This result is in better agreement with experimental data, suggesting that TSC2 might be a better substrate for GSK3 than $\beta$-catenin. Since TSC2 can also be part of the destruction complex [10], this is plausible. Nonetheless, the overall signaling logic of the model was in disagreement with experimental data and this suggested that the model had to be refined.

### 2.2 Second modeling iteration: a model of a detailed Akt-Wnt crosstalk

To model a simple interaction between the Akt pathway and the Wnt pathway was insufficient to reproduce the experimentally observed pattern for the activity of mTOR in neurons. We further looked into the literature for molecular details on the crosstalk mechanism. We first considered the interaction between TSC2 and the destruction complex. It was reported that TSC2 was present in a pulldown of Axin or APC suggesting that TSC2 can be part of the destruction complex through binding [16, 17]. We modified the model accordingly and assumed that GSK3 has access to its substrate TSC2 only when both proteins

are in the complex. It is also known that the inhibitory effect on TSC2 caused by the phopshorylation by Akt of the residue T1462 can result in the binding of TSC2 to a 14-3-3 scaffolding protein, effectively blocking the binding of TSC2 to the destruction complex [3, 20]. Consequently, once TSC2 is phosphorylated by Akt in the model, it can no longer return to the destruction complex. Other experimental results suggested that the Wnt pathway must be activated to allow Akt to phosphorylate GSK3 [5]. In other words, the recruitment of Axin-GSK3 to Disheveled and the concurrent disassembly of the destruction complex [24] make GSK3 vulnerable to the inhibitory phosphorylation by Akt. We modified the mathematical model to take into account all this biological information. This version contains approximately 60 differential equations. The binding affinity between TSC2 and Axin was estimated, otherwise we used all the kinetic parameters from the first model.

The first simulations of the second iteration model did not produce the expected results. The mTOR activity was mostly a flat line. We investigated the dynamics of the model and concluded that the very low APC concentration was a rate limiting variable. This is a known feature of the Wnt pathway and the low concentration of either Axin in xenopus [13] or APC in hepatocyte [21] have been discussed in the literature. Despite these observations, we explored the behavior of the model at higher concentrations of APC. With higher APC concentrations, the response of mTOR was stronger (see Figure 5, on the left). A high concentration value for APC was then used to simulate the model in the three stimulation conditions: with calcium alone, with Wnt alone and with both signals (see Figure 5, on the right). These simulation results show that the activation of both signaling pathways are required to activate mTOR. These last results — the signaling logic and the signal duration of mTOR — were in good agreement with the experimental data.

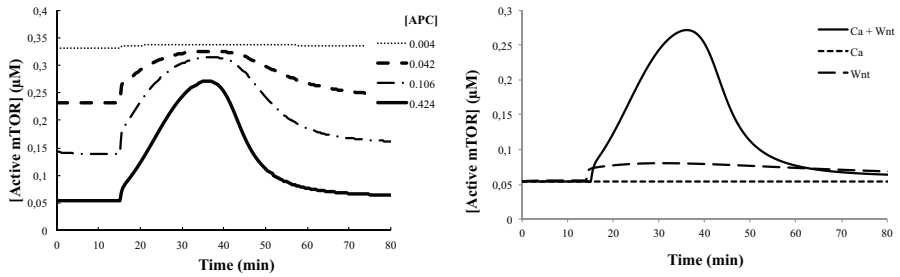


**Fig. 5.** Simulated activation of mTOR in the second iteration model. On the left, simulations with the Wnt stimulation for various concentration values for the protein APC. On the right, simulations with both calcium and Wnt stimulations, with Wnt alone and with calcium alone.

## 3 Petri nets and dynamic graphs of the signaling models

From the simulation results of the ODE models, the source of the duration of the mTOR activity and the signaling logic of the models are unclear. In both cases, the crosstalk between the Akt and the Wnt pathways forms a coherent feedforward motif. This motif can be functionally important in the regulation of the activity of mTOR. Akt directly inactivates TSC2, but it also has an indirect effect by inhibiting GSK3, an activator of TSC2. Furthermore, the phosphorylation of Akt by GSK3 blocks its reintegration in the destruction complex in the first model. Ma et al hypothesized that this might prolong the inactivation of TSC2, thus explaining the long mTOR activity [15]. From the simulation data, this seems unlikely. The second model even seems to suggest the opposite. To get a clearer picture, we created dynamic graph representations of the Akt-Wnt-mTOR model.

The outputs of the simulation of biological systems modeled with differential equations are temporal, and maybe spatial, traces of the different modeled molecular quantities. Ideal to study the dynamic of individual components, the system-level behavior of these models is not easily accessible whenever the models reach a certain level of complexity. To provide a solution to this problem we sought to combine graph theory and dynamical systems into a visualization method: the dynamic graph [6]. We summarize the approach in the next paragraphs.

The dynamic graph uses Petri net theory to bridge two classical approaches: ODEs and graph theory. Using dynamic graphs in a previous project, we understood the dynamics of the signaling network activated by $\beta$-adrenergic receptors in podocytes and correctly predicted from the model the presence of an unknown regulatory mechanism [1]. In this approach, the mathematical equations are converted into a Petri net model of biochemical reactions. Using the SBML import feature of the software Snoopy [8], we created a Petri net representation of the two Akt-Wnt-mTOR ODE models (see Figures 6 and 7)[2].

With the Petri net representation, it becomes possible to analyze the structural properties of the model and extract functional relationships between the variables of the ODEs. These relationships are then used to reconstruct the signaling network underlying the mathematical model. This is done in four steps. First, we identified the marking invariants (also known as P-invariants) using the software Charlie [9]. In biochemical models, these sets of places are known to be associated to mass conservation relationships. In other words, this first level of structural analysis finds variables, which together form linear combinations that are always constant no matter what is the state of the system. In biological models, this is equivalent to a molecule that can change state but whose total concentration stays constant. For example, a P-invariant might represent a protein with its different phosphorylation states or a gene with its on-off conditions.

[2] The models are too large and cannot be read comfortably on paper. However, the files of the Petri net models are available upon request.

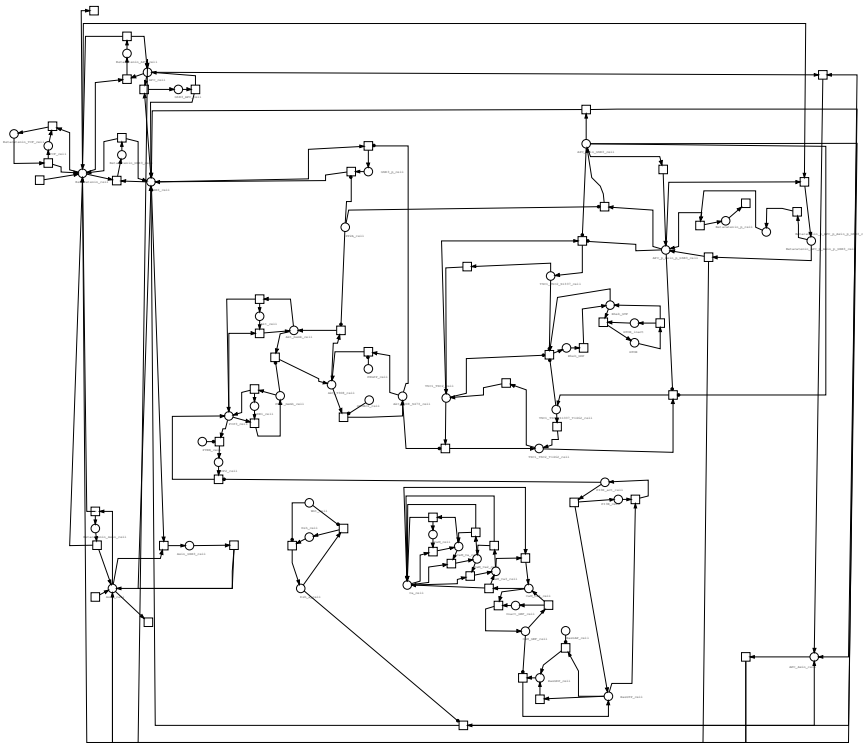

**Fig. 6.** Petri net model of the first iteration Akt-Wnt-mTOR model.

For the second step of the structural analysis, we did not consider the entire Petri net model, but the subnetworks composed of the places in the P-invariant support sets and their connected transitions. For each of these subnetworks, we found the firing invariants (also known as T-invariants) and then grouped the sets that have elements in common. We call these T-invariant supersets signaling segments. The signaling segments divide the network into different "wires" that relay signals: if a signal reaches one place, the signal will consequently perturb all the other places linked to the signaling segment and give way to signal propagation. Thus it is possible to follow the signal as it propagates from one segment to others. Consult [6] for more information on signaling segments.

In the third step of the structural analysis, we proceed to the exploration of the Petri net starting from a predetermined signaling source. In our model, the source is calcium. As we explore the Petri net model jumping from one signaling segment to another, we can build a directed graph where nodes and edges corresponds to P-invariant and signaling segments respectively. Although calcium is biologically responsible for the secretion of Wnt, this relationship is not explicit in the equations, thus a second source appears in the interaction graph. The final step of the structural analysis is to assign an influence type to

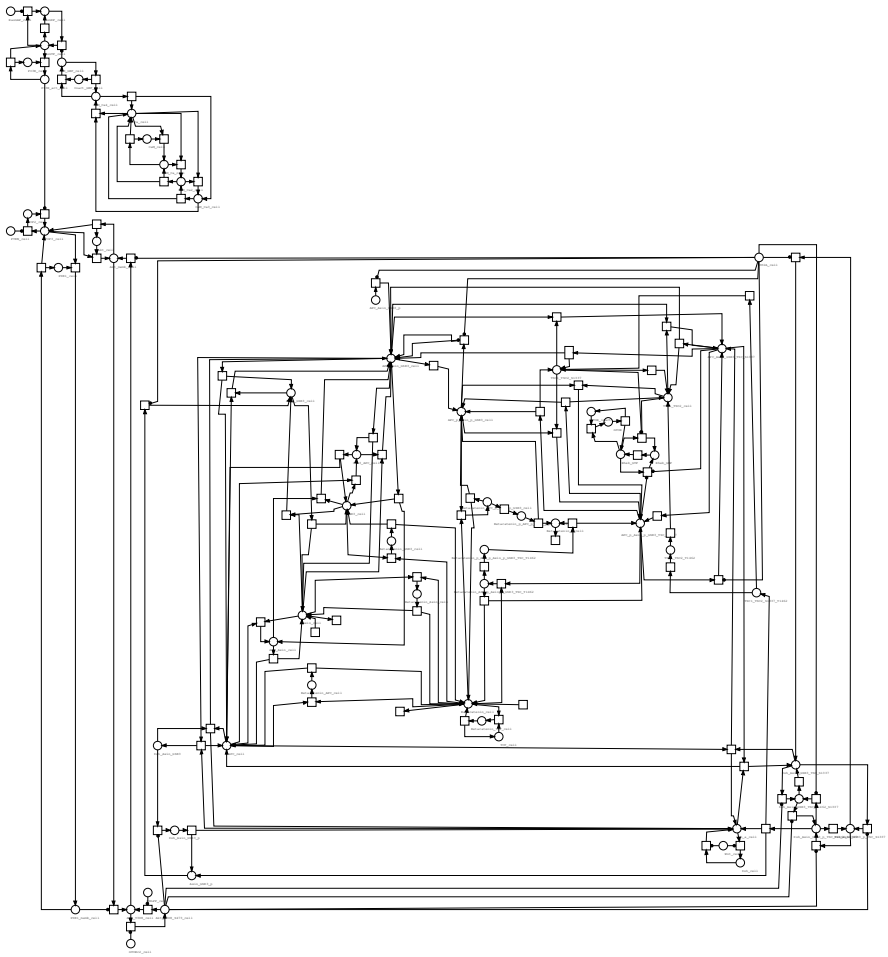

**Fig. 7.** Petri net model of the second iteration Akt-Wnt-mTOR model.

the edges of the interaction graph: a node can either activate or inhibit another node. The assignment of the influence type is done according to a criterion: if the downstream signaling from node Y is initiated or enhanced because of the interaction with node X, then the influence of X on Y is activation, otherwise, it is inhibition. Activation is represented as a regular arrow and inhibition, as a tee shape arrow. The result of this structural analysis is Figure 1. We do not intend to formally present the algorithm to generate an interaction graph in this paper. This work is still ongoing.

The interaction graph generated so far is not a static representation only; it contains all the necessary information to map the simulation data onto the signaling network. To display the activity state of the signaling components of the network, we color code the nodes. A protein is highly active if it is red and

less active as it turns blue. To display the strength of the interactions between signaling components of the network, we color code and modify the thickness of the edges. If a regular (tee) arrow between two nodes is black and thick, then the rate of the activation (inhibition) reactions involving these two proteins is high. When the arrow fades, the reaction rate slows down. This is an indication of the information transfer along the signaling pathway.

For the first iteration Akt-Wnt-mTOR model, we created two dynamic graphs from the simulation data that included the mTOR time courses shown in Figure 4 (left). However, instead of a plot representing the time course of only one variable, the dynamic graphs show the dynamics of 54 variables (places) and 68 reactions (transitions) all at once. The dynamic graph on the left in Supplementary Figure 1 [3] shows the activity of the signaling network when both calcium and Wnt are present. This animation shows an active Akt pathway, reacting to the two calcium influxes, strongly inhibiting TSC2 and eventually causing mTOR to be transiently active. The Wnt pathway is less effective at transmitting the signal to mTOR. The state of GSK3 does not change. This can be explained by the surprising small concentration of the destruction complex. This is a known feature of the Wnt pathway [13]. The Wnt pathway is usually studied for Wnt signals lasting a few hours and causing $\beta$-catenin to slowly increase. For brief stimulations, the low concentration of the destruction complex might act as a signaling bottleneck. The dynamic graph on the right in Supplementary Figure 1 shows that the absence of Wnt does not have a significant effect on the dynamics of the signaling network. Taken together, these graphs show that the feedforward motif (Akt-GSK3-TSC2) of the first iteration model acts as an 'OR' logic gate. Akt by itself can deactivate TSC2, thus activating mTOR as a result. Wnt by itself can deactivate GSK3 although mildly, thus activating mTOR as a result. There is no significant synergy when both pathways are active and the output signal is not prolonged. For these reasons, this model was in disagreement with experimental data.

For the second iteration Akt-Wnt-mTOR model, we created three dynamic graphs from the simulation data that included the mTOR time courses shown in Figure 5 (right). These graphs show the dynamics of 65 variables and 98 reactions all at once. The dynamic graph at the left in Supplementary Figure 2 displays the activity of the signaling network when calcium alone is present. This animation shows an active Akt in response to calcium, but with no effect on mTOR. Both TSC2 and GSK3 are protected within the destruction complex and are still inhibiting Rheb, thus mTOR. The dynamic graph in the middle shows the simulation data when Wnt alone is present. This animation shows a deactivation of the destruction complex, but with little effect on mTOR. Finally, the dynamic graph at the right of Supplementary Figure 2 shows that when calcium and Wnt are triggering signaling together, they strongly activate mTOR. The reason for this response is that the recruitment of Axin to Dsh following its activation by Wnt exposes GSK3 and TSC2 to phosphorylation by Akt.

[3] A version of this paper with the supplementary figures is available at `http://www2.ift.ulaval.ca/~hardy/bioppn2016_hardy.pdf`

This inhibits their signaling activity and as a result, mTOR is activated. Taken together, these graphs show that the feedforward motif of the second iteration model acts as an 'AND' logic gate. Akt and Wnt must be active at the same time for the signal to propagate downstream. This is a coherent feedforward motif with a coincidence detection property. The dual effect of Akt, first on GSK3 and then on TSC2, amplifies also the upstream signal on the downstream target.

## 4 Discussion and conclusion

A major goal of systems biology is to bridge molecular components and higher-level biological functions across multiple scales by understanding the complex interactions in between. To gain the knowledge necessary to control these systems, with therapeutics for example, the topological reconstruction of the underlying networks is insufficient. It is necessary to mechanistically determine the systems dynamics. Regulatory motifs are significant functional modules of cellular machinery that is organized into interconnected networks. The most common ones are signaling, genetic regulation, and metabolism, but include others like epigenetic regulation. Determining the dynamics of these networks is the key to understanding systems complex behavior. To achieve this understanding it is crucial to combine experimental methods with tools from computational cell biology.

In this paper, we presented two dynamic models and their simulation data to analyze the signaling dynamics of the network regulating the protein mTOR, an important regulator of cellular protein synthesis. We showed early work on an evolving mathematical model of two pathways known to be upstream of mTOR: the Akt and Wnt pathways. An initial version of the model was assembled from the combination of two published models [11,21]. This model was then modified to include more molecular details from the literature. The dynamic behavior of our second model relies on the fact that GSK3 and TSC2 are protected from the inhibitory phosphorylation of Akt when in the destruction complex. Simulation results and analysis with dynamic graphs of the second model showed that the crosstalk between the two pathways forms a regulatory motif that functions as a coincidence detector. These results are in agreement with published data [15]. Another interesting preliminary result of this theoretical study is that the concentration of the protein APC must not be at low levels to support this function contrary to what was observed in other species and cell lines for this protein. Some evidence in early measures of APC in neurons already supports this hypothesis [2]. This is a prediction that can be tested experimentally.

We still cannot provide a satisfying answer to the initial question that motivated this study: How is it possible that a strong, but very brief, synaptic stimulation can trigger the prolonged activation of mTOR needed to induce L-LTP? The biological signal tends to slow down as it propagates downstream. The sudden production and realease of biomolecules like PIP3 and Wnt and their slow degradation contributes also to lengthen mTOR activity. Nonethe-

less, we are still short of at least 15 minutes of activity, which represents 33% of the time mTOR is active after HFS stimulation. Our results also show that the Akt-GSK3-TSC2 feedforward motif cannot completely assume this function. This suggests that the network is incomplete and that other network motifs are probably involved. In the next phase of this project, we will add other proteins and interactions to the model. We will take a closer look at the role of ERK since it is activated by calcium and is involved in the regulation of Akt and p70S6K, a substrate of mTOR. We will also explore the possibility that one or more feedback loops might modulate mTOR activity. For example, ERK and Wnt are part of a positive feedback loop in metastatic cells [12]. All these possibilities can be tested with modeling and simulation to provide valuable insights to experimentalists and identify the most promising hypotheses.

Petri nets are an essential tool in our approach of theoretical analysis of biological systems. They provide a link between dynamical formalisms and graphs. In this paper, the use of dynamic graphs was limited, but its role will become substantial as the model grows in complexity and in number of interacting network motifs. Dynamic graphs provide a holistic view of the system behavior. We are currently working on the algorithm that will generate dynamic graphs for models with complex biochemical mechanisms, such as the destruction complex regulation by Wnt. We also plan to explore the use of formal methods to detect and characterize interactions in dynamic graphs to go beyond visual analysis. Potential uses for this last feature are a tool to objectively analyze complex behaviors between different versions of a model, for example when exploring the parameter space, or an investigation tool into the dynamics of models of very large biological systems.

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