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The Role of Retinoic Acid and Notch in the Symmetry Breaking Instabilities for Axon Formation

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Abstract: A mathematical model analogous to a devised model of biological pattern formation (Rauch and Millonas, 2004) is proposed to investigate the effects of Retinoic Acid(RA) and activated Notch1 (i.e., Notch1 intracellular domain, NICD) on neurite outgrowth in N2a cells. The model consists of reaction-diffusion systems with feedback loops. Here we consider RA as an external signal with a positive feedback and activated Notch as an inhibitory signal in a negative feedback loop. In our model the perturbations introduced by diffusion terms destabilize the balance between the positive and the negative feedback loops. Consequently, the symmetry between the neurites (transmembrane proteins) breaks and one of them starts growing faster than the other neurites (Andersen and Bi, 2000). The conditions for the existence of symmetry breaking instabilities are established by linear analysis. We show that these conditions are dependent on the strength of the feedback loops. We hypothesize an interaction between Notch and RA signaling pathways. This interaction is considered as a perturbation to the systems. On the basis of numerical results, we present a bifurcation analysis for the cases of perturbed and unperturbed systems. Numerical solutions are presented and used for further predictions. The analytical and numerical outcomes of the model are explored along with laboratory experimentations.

Key-Words: neurite outgrowth, neuroblastoma, symmetry breaking, feedback loops, Retinoic acid(RA), Notch

1. Introduction

The nervous system is developed as a result of an orchestrated series of cell division, cell fate commitment and differentiation that give rise to specific cell types called neurons and glia. Each of these cell types demonstrates a specific structure. In particular, a neuron consists of a single axon (signal transmitter), a nucleus and a host of dendrites. A key question in Mathematical Biology is how the fate of an axon is determined. It has been suggested that all processes have an equal potential to become an axon prior to neuronal differentiation (Dotti and Banker, 1988). But only one of the processes becomes committed to an axonal fate. In a proposed molecular model of axon formation (Anderson and Bi, 2000), it has been suggested that the regulation between the positive and negative feedback loops provides a robust mechanism for spontaneous symmetry breaking and formation of only one axon. This occurs when the symmetry between the neurites (transmembrane proteins) breaks and one of them starts growing faster than the other neurites. It is known that Notch signals can antagonize neurite outgrowth in neuroblastoma cells (Franklin et al., 1999) whereas experiments demonstrate that Retinoic Acid(RA) promotes neurite outgrowth in neuroblastoma cells. Here we consider RA as an external signal with a positive feedback and activated Notch as an inhibitory signal in a negative feedback loop. In our model, the perturbations by diffusions destabilize the balance between the positive and the negative feedback loops and the symmetry is broken.
Lateral inhibition is a type of cell-cell interaction

whereby a cell that adopts a particular fate inhibits adjacent cells from acquiring the same fate. This is controlled by a negative feedback loop: the more inhibition a cell delivers to its neighbors, the less it receives back from them and the more it is consequently able to deliver (Wearing et al., 2000). The mechanism 'lateral inhibition with feedback' has been used in modeling Delta-Notch signaling for biological pattern formation and cell fate determination (Collier et al., 1996). Collier and his colleagues showed that the initial slight difference of the level of Delta and activated Notch between the neighbors will become self-amplifying, generating a full-blown spatial pattern of inhomogeneity. Correspondingly, there is evidence that N2a neuroblastoma cells with high levels of Delta activity and low levels of Notch activation become neurons while cells with low Delta activity and high Notch activation levels remain undifferentiated (Franklin et al., 1999). This suggests that the mechanism of lateral inhibition with feedback can be used to enlighten the regulation of neurite outgrowth in N2a neuroblastoma cells. Turing (Turing, 1952) showed that chemicals can react and diffuse in such a way that spatial patterns of concentration are established and as a consequence of this, the fate of a cell is determined. There are a growing number of articles suggesting the realistic relevance of Turing mechanism to spontaneous symmetry breaking (see Sawai et al., 2000, for example). In both the Turing mechanism and feedback mechanism, the pattern formation happens when there are instabilities to the small perturbations. The assumption is that there is a direct relation between the strength of the feedback loops and the diffusion of the signaling molecules, so that the perturbations by diffusion can be interpreted as the perturbations by feedback loops and vice-versa. The phenomenon of Turing instability has been widely used in many branches of biology. A recent interesting approach to Turing instability is proposed in a model wherein activator and inhibitor are included into the biochemical context (Rauch and Millonas, 2004). In fact, for the first time, a network of signaling pathways is added to the Turing mechanism. The present work utilizes the same approach for the production of a broken spatial symmetry. We develop a model analogous to their devised model to investigate the effects of RA and activated Notch on neurite outgrowth and neuronal differentiation. In Section 2, we introduce our model and describe the main assumptions. In Section 3, we use linear stability analysis to derive the conditions for the existence of Turing instabilities. We provide a two-cell system analysis similar to the work by Collier et. al (1996) In Section 4, on the basis of numerical results, we present a bifurcation analysis for the cases of perturbed and unperturbed systems. We demonstrate that conditions for pattern formation depend on the strength of the feedback loops. And finally in Section 5, we submit our conclusions.

2. The Mathematical Model

The present model extends that proposed by Rauch and Millonas (2004) in two important respects. First, it takes into account the essential role of nonlinearity in the equations representing the transformation of activator and inhibitor into corresponding signaling molecules and the reverse transformation of the molecules into activator and inhibitor. Secondly, it is well known that lateral inhibition plays a key role in pattern formation and cell fate determination (Lewis, 1998; Collier et al., 1996; Owen et al., 1999). The influential mechanism of lateral inhibition is a crucial factor in a system of feedback loops that we consider in our model. The Model we present here, embodies the following assumptions:

1. Cells interact through feedback loops only with their adjacent cells.
2. The strength of feedback loops can be affected by external signals: activated Notch weakens the negative feedback and RA signals strengthen the positive feedback.
3. The symmetry breaks only when a feedback gets stronger and the balance between the feedbacks becomes unstable.
4. The level of activated Notch and the concentration of RA in a cell determine cell differentiation: low levels of Notch and high concentrations of RA lead to neuronal differentiation, otherwise a cell remains undifferentiated.
5. The system is perturbed by interactions between Notch and RA signaling pathways: NICD slows down RA signals by blocking Retinoic Acid receptor (RAR) in the nucleus and in a set of reactions RA catalyzes the production of more inhibitor (Notch).

The elements of the model are activated Notch protein (v), the level of Delta activity (w), concentration of RA in each cell (u) and the level of microtubule

associated protein 2 (MAP-2) activity (q) in terms of local polymerization in each cell. In terms of activator and inhibitor, Notch is the inhibitor and Delta is the activator. Also RA is considered as the second activator in our model. Here, RA is an external signal with a positive feedback such that it catalyzes the polymerization of MAP-2. Notch-RA interaction is considered as a perturbation to the system.The non-dimensionalized form of the model is as follows:

$$u_t = \gamma_1 \left(f_\alpha(u) + \epsilon s(u,v)\right) + d_1 \nabla^2 u \quad (1)$$

$$v_t = \gamma_2 \left(g_\alpha(v) + y(w) + \epsilon \overline{s}(u,v)\right) + d_2 \nabla^2 v \quad (2)$$

$$w_t = \gamma_3 \left(-a_3 w + z(v)\right) + d_3 \nabla^2 w \quad (3)$$

$$q_t = \gamma_4 \left(u - a_4 q\right) + d_4 \nabla^2 q \quad (4)$$

where all the constants γ_1, γ_2, a_3,... are positive; Diffusive transport of external signals and also transport of proteins between the segments of the same cell is included to the system. Coefficients $d1, d2, d3$ and $d4$ are the rate of diffusion related to each component. $\epsilon > 0$ is a small (perturbation) parameter which represents the interactions between the external positive signal(RA) and the inhibitory signal(Notch). $\alpha \in (0,1)$ is a (bifurcation) parameter which is related to the concentration of RA and the level of activated Notch utilized in the experiment.

As mentioned above, Delta and Notch interact in a negative feedback loop. Here, we take y and z to be in the same form as they are proposed in previous articles(e.g. Collier et al., 1996). $y, z : [0,\infty) \to [0,\infty]$

$$y(x) = \frac{x^k}{c_2 + x^k} \quad (5)$$

$$z(x) = \frac{1}{1 + c_3 x^h} \quad (6)$$

where $c_2, c_3 > 0$ and $k, h \geq 1$ with the boundary conditions zero Delta activity and zero RA activity. The parameter values we use here to generate the illustrations are $k = h = 2$.

Functions f_α and g_α represent the kinetics of RA and Notch signals in the absence of feedback loops. It is known that RA induces neuronal differentiation in many types of cells (see Napoli, 1996 for example). It is also known that Notch signals can antagonize neurite outgrowth in neuroblastoma cells (Franklin et al., 1999). These are two important factors which are reflected in our model in the following sense:

The level of activated Notch utilized in the experiment is proportional to the parameter α, while concentration of RA is proportional to $\frac{1}{\alpha}$. We take the functions f_α and g_α in the following forms:

$$f_\alpha(x) = \frac{1}{\alpha} - a_1 x \quad (7)$$

$$g_\alpha(x) = \frac{a_2}{(\alpha - 1)}(x - \alpha)^2 \quad (8)$$

where $\frac{1}{\alpha}$ is the concentration of RA added to the system in each experiment and a_1 is the rate of removal.

An interaction between Notch and RA signaling pathways is considered as a perturbation to the system. In order to investigate the effects of this interaction on the system , we introduce functions s and $\overline{s}$ in the following forms:

$s(u,v) = -c_1 v$ and $\overline{s}(u,v) = l_2 u$ where $\overline{s}$ represents that RA catalyzes production of more Notch and s represents that Notch suppresses production of RA.

3. Linear Stability Analysis of the System

We begin our study of the pattern-forming potential of our model by analyzing the stability of the homogeneous steady states. We set the coefficients of our system of equations (1)-(4) to: $a_1 = a_2 = l_2 = c_1 = 1$; $c_2 = 10\alpha$, $c_3 = 100\alpha$. It is not difficult to see that the system admits a steady state for a suitable choice of α. By linearizing the system about the steady state $(u_\epsilon, v_\epsilon, w_\epsilon, q_\epsilon)$ in a usual way (Murray, 2003), we get the following stability matrix:

$$A_\epsilon = \begin{pmatrix} \gamma_1 f_u & -\epsilon\gamma_1 & 0 & 0 \\ \epsilon\gamma_2 & \gamma_2 g_v & \gamma_2 y_w & 0 \\ 0 & \gamma_3 z_v & -\gamma_3 a_3 & 0 \\ \gamma_4 & 0 & 0 & -a_4\gamma_4 \end{pmatrix}$$

where A_ϵ is the coefficient matrix associated with the linearized system near the steady state. Here we present the necessary and sufficient conditions for Turing instability of the steady state for the case $\epsilon = 0$, while for $\epsilon > 0$, a perturbation analysis is required which can be another case of study. The necessary conditions for Turing instability are presented in inequalities (9) and (10):

$$P_0 : \gamma_2 g_{v_0} - a_3\gamma_3 < 0 \quad (9)$$

$$Q_0 : a_3 g_{v_0} + y_{w_0} z_{v_0} < 0 \tag{10}$$

By taking zero flux boundary conditions and given initial condition for equations (1)-(4), we obtain the sufficient conditions (when $\epsilon = 0$):

$$\left| A_\epsilon - \lambda I - k^2 D \right| = 0 \tag{11}$$

$$T_0 : d_c \gamma_2 g_{v_0} - \gamma_3 a_3 > 0 \tag{12}$$

$$-\gamma_2 \gamma_3 Q_0 < \frac{T_0^2}{4 d_c} \tag{13}$$

where D is the diagonal matrix of diffusion coefficients, k corresponds to wave number and $d_c = \frac{d_3}{d_2}$ is the diffusion ratio.

Fig. 1 shows the effect of varying ϵ on the Turing instabilities as $\epsilon \to 0$ the range of pattern formation increases. Fig. 2 is the Plot of the largest of the eigenvalues $\lambda(k^2)$. For several values of parameter α. the system admits Turing-type patterns when $0.37 < \alpha < 0.58$

For a system consisting of two cells with periodic boundary conditions and $\epsilon = 0$, by equations (2) and (3) in our model we get:

$$\dot{v}_1 = \gamma_2(g_\alpha(v_1) + y(w_2)), \dot{w}_1 = \gamma_3(-a_3 w_1 + z(v_1), \tag{14}$$

$$\dot{v}_2 = \gamma_2(g_\alpha(v_2) + y(w_1), \dot{w}_2 = \gamma_3(-a_3 w_2 + z(v_2)), \tag{15}$$

where the subscripts correspond to cells 1 and 2. Let:

$$Z(x) = \frac{1}{a^3} z(x) \tag{16}$$

$$G(x) = \alpha + \sqrt{(1-\alpha) y(x)} \tag{17}$$

then v_1 and v_2 are the fixed points of the composition function $GZGZ$. And the system of two cells is unstable if we have $(GZGZ)'(v_1) > 1$. (18)

Since GZ is monotonic decreasing, there exists $x_0 \epsilon [0, GZ(0)]$ such that $x_0 = GZ(x_0)$ and x_0 is the unique fixed point of GZ. Hence, the steady states of the two-cell system must have unique components $(v_1, w_1, v_2, d_2) = (x_0, z(x_0), x_0, z(x_0))$. if $(ZG)'(x_0) < -1$, then there must be at least one period 2 solution of map.It can be seen that $(ZG)'(x_0) < -1$ is equivalent to instability condition (18). Therefore in a Two-cell system existence of an unstable homogeneous steady state corresponds to a pair of heterogeneous steady states. Consequently, one of the cells can differentiate to a neuron and the other cell remains undifferentiated.

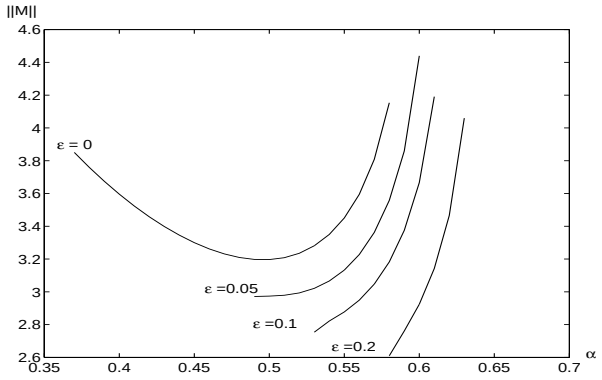

Figure 1. Plot of Turing instabilities for different values of $\epsilon > 0$. As $\epsilon \to 0$ the pattern formation may happen in a wider range of α.

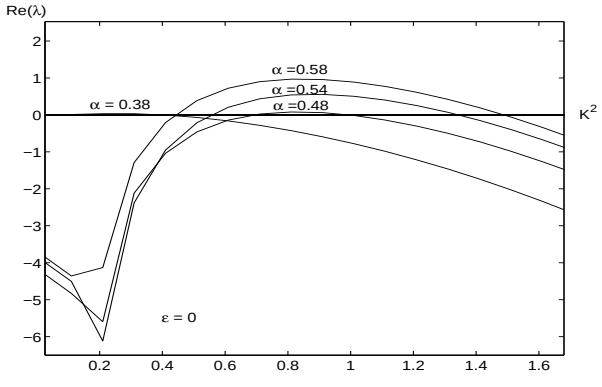

Figure 2.Plot of the largest of the eigenvalues $\lambda(k^2)$.the system admits Turing-type patterns when $0.37 < \alpha < 0.58$.'k' corresponds to wave number

4.Numerical results and Bifurcations

The Turing bifurcation is the basic idea for generation of spatial patterns which can be found in most of the mathematical models for biological pattern formation. The bifurcation we are interested in here is a different one. We are concerned with strength of feedback loops. The balance between positive and negative feedback becomes unstable when a feedback gets stronger and eventually the symmetry breaks (Andersen and Bi, 2000). Mathematically, this event corresponds to a bifurcation where changing a parameter in the system leads to a possible qualitative

change in the stability of the steady states or they bifurcate at a certain point.

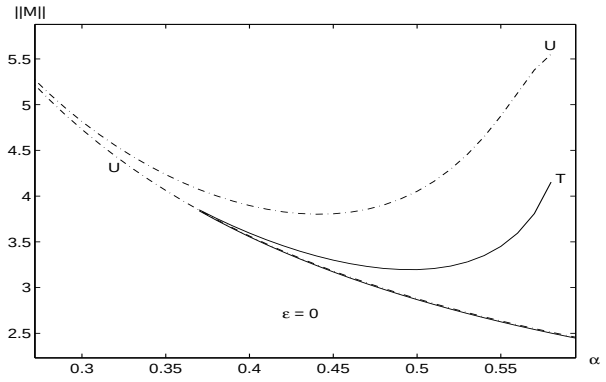

Figure 3. Bifurcation diagram for $\epsilon = 0$. T= Stable steady states with Turing instabilities, U= Unstable Steady States , Turing instabilities occur in the presence of Unstable steady states which in a two-cell system corresponds to pattern formation

In a one dimensional Delta-Notch system where we have a line of cells, it has been shown that the homogeneous steady state becomes unstable when the negative feedback is sufficiently strong (Collier, 1996). Consequently, the steady state bifurcates into a pair of inhomogeneous steady states such that one(cell) has high Notch activity and low Delta activity (the undifferentiated cell), while the other has high Delta activity (symmetry breaks and the cell becomes a neuron). The fact that Notch signals antagonize neurite outgrowth and RA signals promote neuronal differentiation can be used in our model in the following sense: When activated Notch is utilized we have a negative external signal in our system which weakens the strength of the negative (Delta-Notch) feedback loop. Also, a higher concentration of RA in each cell results in stronger positive feedback within our system. Equations (1)-(4) can be written in the form $\frac{dX}{dt} = F(\alpha, \epsilon, X)$ where $X = (u, v, w, q)$ and $\epsilon \geq 0$ is the perturbation parameter and $\alpha \in (0, 1)$ is the bifurcation parameter. Steady states of the system are presented by $X(\alpha, \epsilon)$ where all components of $X(\alpha, \epsilon)$ must be positive. By solving $F(\alpha, 0, X) = 0$ one can observe that the unperturbed system $(\epsilon = 0)$admits a saddle-node bifurcation, where numerical results reveal that α = 0.3625 is the saddle-node bifurcation value. The pair of saddle and node steady states exists for the values of $\alpha \in (0.3625, 0.589)$ where surprisingly the node steady state satisfies all conditions for Turing instability.

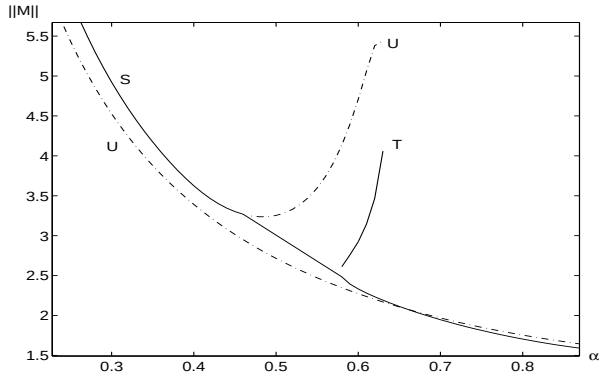

Figure 4. Bifurcation diagram for $\epsilon > 0$. T= Stable steady states with Turing instabilities, U/S= Unstable/Stable Steady States ,Again Turing instabilities occur in the presence of Unstable steady states

In the framework of Turing theory there is a high potential of pattern formation and consequently neuronal differentiation for the cells having the values close to the components of the node steady state.Numerical results show that in the node steady states the level of Notch activity is high while the level of RA concentration is very low. When the system is perturbed $(\epsilon > 0)$ we get the same results, but in a shorter range for α (for example $\epsilon = 0.05$ $\alpha \in (0.48, 0.60)$). This suggests that for $(\epsilon > 0)$ small enough the perturbed system could be topologically equivalent to the unperturbed system. However, this is not the focus of this article. Figures 3 and 4 show the bifurcation diagram for perturbed and unperturbed systems. In the region where saddle steady states exist , the two-cell analysis suggests that there is a high potential of pattern formation and neuronal differentiation.For $\epsilon > 0$ Turing instabilities occur in the same region with a shorter range of α

Adding RA with a lower (higher) concentration and using a higher (lower) level of activated Notch in experiments are subject to an increase (decrease) in the value of α in our model. In fact $f_\alpha \to \infty$ as $\alpha \to 0$shows that the positive feedback function gets ultimately strong and $g_\alpha \to -\infty$ as $\alpha \to 1$ shows that the negative feedback function gets ultimately weak. Figure 5 shows the effects of RA and / or activated

Notch on the morphology of N2a cells , where one can see an increase to the concentration of RA results more differentiated cells (formation of more Axons),while utilizing a high level of activated Notch results most of the cells undifferentiated (Axon formation only in few cells)

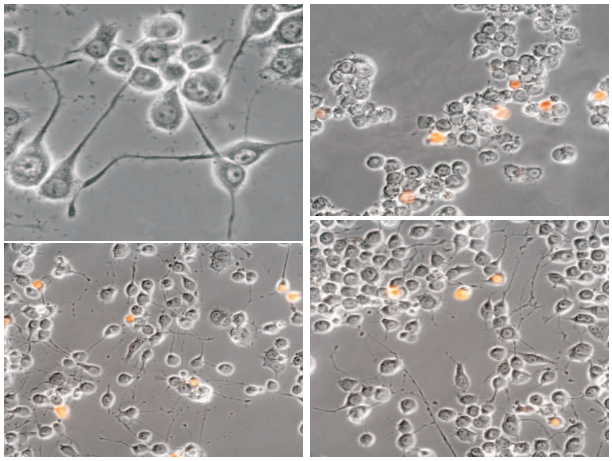

Figure 5. the effects of RA and / or activated Notch on the morphology of N2a cells Top left: In presence of RA (10^{-2}) Top right: In presence of Notch 1 ICD Bottom left: In presence of RA (10^{-4}) and Notch 1 ICD Bottom right: In presence of RA (10^{-2}) and Notch ICD

5. Conclusions

The proposed model is another example of including a network of signaling pathways into the Turing mechanism. We speculate that small perturbations of interaction between signaling pathways doesn't have a qualitative change to Truing instabilities. Experimental results confirm that high concentrations of RA with low levels of activated Notch lead to neuronal differentiation (axon formation) where in theory this is corresponding to existence of Turing instabilities and heterogeneous steady states.It is felt that one particular merit of the work presented here is that it shows the possible existing connection between feedback mechanism and Turing mechanism.Previous work has shown that pattern formation can occur when the homogeneous steady state is unstable (Collier, 1996).We find that Turing instabilities occur in a range where there exist unstable steady states. This suggests that feedback mechanism and Turing mechanism provide similar results for pattern formation.

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