Instabilities in Cellular Dendritic Morphogenesis

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Neuronal dendritic arbors grow in a manner consistent with the concept that cytoskeletal polymerization is catalyzed by the local submembrane concentration of a morphogen believed to be the calcium ion. Linear stability analysis of such diffusion-controlled growth through voltage-gated membranes coupled to active extrusion suggests that dendritic arboring will occur provided the cell is large enough and the membrane is excitable. Numerical simulations confirm this prediction.

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Investigations since the times of Ramon y Cajal [1] have uncovered an abundance of neuronal types, distinguished mainly by the shapes of their dendritic arbors. More recently, it has also been suggested that dendritic arbors exhibit self-similarity over a limited range of scales [2–4], with a fractal dimension consistent with diffusion-limited growth [5]. Fractal dimensions d_f have been found for a number of approximately flat neuronal arbors: for cat retinal ganglion cell dendrites [2,3], $d_f \approx 1.68 \pm 0.15$ or $d_f \approx 1.73$, while for mouse cerebellar Purkinje cell dendrites [4] $d_f \approx 1.71$, all close to those associated with two-dimensional diffusion-limited growth.

But if neuronal dendritic growth is a diffusive process, it is unlike diffusion-limited growth in at least three regards: (i) the nature of the growth mechanism itself; (ii) the fact that cells are bounded by membranes which may be excitable; and (iii) the existence of active pumping mechanisms that can admit growth-regulating substances or extrude them against their concentration gradients.

First, neuronal growth is a dynamic process in which both growth and retraction can be observed, in itself at variance with usual diffusion-limited growth. This growth is not simply an interfacial process involving membrane addition. Rather it represents a complex dynamics in the cell interior in which cytoskeletal polymerization and depolymerization processes occur [6] involving tubulin, actin, and other polymers.

There are persuasive indications that these reaction kinetics are catalyzed by a diffusive morphogen, and that calcium ion is such a morphogen, playing a central role in the regulation of neurite outgrowth [7]. Intracellular free calcium levels in growing neurons are high in regions of outgrowth and in motile growth cones [8]; neurite outgrowth can be inhibited by treatments that either decrease or greatly increase the intracellular calcium level [9]; and it is known that growth cones bear calcium channels [10] that could mediate these effects [11]. On the basis of these observations, it has been inferred that extension of the neurite growth cone depends in a bell-shaped fashion upon its submembrane calcium concentration: Outgrowth increases as the calcium concentration rises to some optimum level, and progressively decreases at higher levels [7]. To model this bell-shaped dependence of outgrowth and retraction upon the submembrane calcium concentration $C(s,t)$ at a point s on the interface at time t, we can parametrize the growth velocity normal to the surface at this point as

$$V(s,t) = V(C(s,t)) + W(t), \tag{1}$$

where $V(C(s,t)) = k_1 C(s,t)^{\alpha} - k_2 C(s,t)^{\beta}$, and k_1 and k_2 are lumped rate constants for the growth and retraction mechanisms, respectively, while the magnitudes of α and β reflect the cooperativity of these polymerization mechanisms' dependence on calcium. The term $W(t)$ is chosen so that the global addition of material to the cell is constant in time.

Second, the membrane may be excitable. Biological membranes can be considered to be phospholipid bilayers covered by ionic channels created by membrane spanning proteins whose conformation depends on the transmembrane potential ϕ. In the resting state this transmembrane potential is nonzero, typically at a potential of about $\phi_{\text{rest}} \approx -60$ mV with respect to the exterior because of the disparity in ionic concentrations on the two sides of the membrane ($[Na^+]_{out} \approx 140mM$, $[Na^+]_{in} \approx 10mM$; $[K^+]_{out} \approx 4mM$, $[K^+]_{in} \approx 120mM$; $[Cl^-]_{out} \approx 120mM$, $[Cl^-]_{in} \approx 12mM$; $[Ca^{++}]_{out} \approx 2mM$, $[Ca^{++}]_{in} \approx 10^{-4}mM$ or $100nM$). This resting potential is set mainly by K^+ because of its high permeability: K^+ ions will tend to diffuse down their concentration gradient out of the cell leaving a submembrane concentration of negative ions, and consequently a negative resting potential at the Nernst equilibrium. As the membrane depolarizes (becomes less negative), the voltage-gated ion channels open. As the transmembrane potential is itself a nonlinear function of the ionic concentrations in the internal and external

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domains, this can result in an effective diffusive morphogen current $j_{\rm diff}(C(s))$ which increases with the local submembrane morphogen concentration $C(s)$. A positive feedback mechanism for cytoskeletal polymerization would thus exist which, we argue, leads to dendritic cellular growth.

For real biological membranes the transmembrane potential is set by all the ionic concentrations. The instability can, however, be discussed in the context of a simpler situation where the resting potential is modulated mainly by the submembrane morphogen concentration $C(s)$ taken to be the Ca^{++} ion, and therefore electrical potential variations $\phi(s)$ across the membrane reflect these internal ionic morphogen variations. For example, suppose the local ionic calcium flux into the cell is approximated by the Ohmic form

$$j_{\rm diff}(\phi(s)) \approx g(\phi(s))[\phi_{\rm Ne}(s) - \phi(s)]/q, \qquad (2)$$

where $g(\phi)$ is the voltage-gated calcium conductivity; $\phi_{\rm Ne}(s) = (kT/q)\ln[C_{\rm out}/C(s)]$ is the local Nernst potential for calcium in terms of its charge q and concentrations $C(s)$ inside and $C_{\rm out}$ outside the cell (taking the outside of the cell to be well mixed, an assumption in accord with the observation that normal development appears in neurons grown *in vitro* in the presence of well mixed external media). The transmembrane potential is then approximately $\phi \approx [g_l\phi_{\rm rest} + g(\phi(s))\phi_{\rm Ne}(s)]/[g_l + g(\phi(s))]$, lumping together all contributions from the other ionic fields into a leakage conductivity g_l and a resting potential $\phi_{\rm rest}$. From these equations one can show that provided the inequality $\partial g/\partial\phi > [g_l + g(\phi)]^2/g_l(\phi_{\rm rest} + \phi_{\rm Ne})$ is obeyed, $\partial j_{\rm diff}/\partial C > 0$ and a positive feedback mechanism exists.

Third, active pumping of ionic species against their diffusive gradients is a necessary condition for living cells to exist (otherwise death would occur on diffusive time scales). Specifically the internal cellular calcium concentration $[Ca^{++}]_{\rm in} \approx 100nM$ in the resting state is about 4 orders of magnitude lower than the external concentration $[Ca^{++}]_{\rm out} \approx 2mM$. Such gradients can only be maintained by energy (ATP)-dependent pumping mechanisms which experimentally are found to be highly nonlinear, and to a good approximation sigmoidal [12] functions of the local ionic concentrations. For calcium, active pumping sets in when the internal calcium concentration rises to a critical value $C_{\rm crit} \approx 1000nM$, and therefore we may parametrize the pumping locally as

$$j_{\rm pump}(C(s)) = j_{\rm max}/(1 + \exp\{[C_{\rm crit} - C(s)]/\Delta C\}). \qquad (3)$$

Combining these observations, the assumption in this Letter is that the rate of cellular dendritic growth is determined locally by the internal submembrane concentration of a diffusible morphogen $C(s)$, assumed to be the calcium ion, which is involved in the catalysis of cytoskeletal polymerization and depolymerization kinetics. Its concentration, in the quasistatic approximation, involves the solution of Laplace's equation $\nabla^2 C = 0$ subject to biologically plausible boundary conditions for the morphogen flux which can be found by equating the diffuse flux just internal to the cell to the total flux by all mechanisms through the membrane

$$-D\nabla C(s) = j(C(s))\mathbf{n}(s). \qquad (4)$$

Here $j(C(s)) = j_{\rm diff}(C(s)) - j_{\rm pump}(C(s))$ is the total flux through the membrane due to both diffusion $j_{\rm diff}(C(s))$, a function of the submembrane calcium ion concentration which modulates the local conductivity and electrochemical gradient across the cell membrane such as Eq. (2), together with is active extrusion by pumping $j_{\rm pump}(C(s))$ against that gradient given by a form such as Eq. (3), while $\mathbf{n}(s)$ is the local normal into the cell at a point s. A drift contribution $\sigma\nabla\phi$ to the total flux has been neglected [$|\sigma\nabla\phi/qD\nabla C(s)| \sim q\Delta\phi/kT$] in Eq. (4) as the typical spatial variation in transmembrane potential across the cell $\Delta\phi$ is small (a few mV) compared to the diffusive contribution.

Dendritic growth requires a spatially varying morphogen concentration. This could occur in one of two ways in an initially circular or spherical cell. Either surface fluctuations perturb the morphogen fluxes through the membrane resulting in submembrane concentration fluctuations which lead to unstable growth, or a constant ionic concentration inside a growing cell is unstable in the presence of a voltage-gated membrane leading to spatially inhomogeneous concentrations, and endogenous currents (the Pelce instability [13]); if these currents are morphogens, dendritic growth may result.

Consider first the case of the surface instability. To study its linear stability we use the geometric approach introduced by Brower, Kessler, Koplik, and Levine [14] for the motion of curved fronts, generalizing the dynamics to the case of excitable membranes growing in response to a morphogen field $C(s,t)$. The local curvature $\kappa(s,t)$ of the membrane at a point s on the membrane surface will change with time as

$$d\kappa(s,t)/dt = -[\partial^2/\partial s^2 + \kappa^2][V(s,t) + \gamma\partial^2\kappa/\partial s^2], \qquad (5)$$

with an effective growth velocity $V(s,t)$ which we take to be dependent on calcium in a bell-shaped manner such as Eq. (1). We have also included an effective membrane rigidity [15] using the free energy $F_{\rm rigid} = \gamma\int_0^L ds(\partial\kappa/\partial s)^2$ which will discourage noncircular growth, and whose value reflects not only the bending rigidity of the phospholipid membrane but is also set by the rigidity of the internal cytoskeleton.

The unperturbed growth is taken to be circular, in which case $\kappa(s,t) = R_0(t)^{-1}$, and the unperturbed growth is simply $R_0(t)^2 = R_0(0)^2 + kt$ from conservation of mass, where k is proportional to the global rate of cytoskeletal polymerization. Assume a spatially homogeneous morphogen flux j is flowing through the membrane. Then the internal morphogen concentration during

this unperturbed growth is $C_0(r,t) = C'(R_0)\ln(r/a)$, where $C'(R_0)$ is the solution of the implicit equation $DC'(R_0)/R_0 = j(C'(R_0)\ln(R_0/a))$. Here we have assumed that internal buffering keeps the morphogen concentration at very low levels, $C(a) \approx 0$, close to the center ($r = a$) of the cell. To look for the linear stability of these growing circular solutions, we infinitesimally perturb the surface

$$R(\theta,t) = R_0(t) + \sum_{m=1}^{\infty} \delta_m(t)\cos(m\theta), \tag{6}$$

where θ is the azimuthal angle. In turn these surface fluctuations perturb the calcium concentration, which to first order in these perturbations can be written

$$C(r,\theta,t) = C'(R_0(t))\left(\ln(r/a) + \sum_{m=1}^{\infty} a_m(t)[\delta_m(t)/R_0(t)]\cos(m\theta)[(r/a)^m - (r/a)^{-m}]\right), \tag{7}$$

with $a_m(t) = [D/R_0(t) + \partial j/\partial C_0](R_0/a)^{-m}/[mD/R_0 - \partial j/\partial C_0]$ provided $R_0/a \gg 1$ and $(dC'/dR_0)/C' \ll 1/R_0$. Substitution of these expressions into Eq. (5) results in the growth of the perturbations as

$$d\delta_m/dt = (1 - m^2)\delta_m[m^2\gamma/R_0^4 + X], \tag{8}$$

where $X \equiv [C'(R_0)/R_0]\partial V/\partial C_0\{1 + [1 + (R_0/D)\partial j/\partial C_0]/[m - (R_0/D)\partial j/\partial C_0]\}$. An examination of Eq. (8) reveals some of the important factors influencing cellular dendritic growth. The membrane must be excitable $\partial j/\partial C_0 > 0$; indeed the neuron must achieve a minimum size

$$R_{\min} = 2D/(\partial j/\partial C_0) \tag{9}$$

before dendrites can emerge.

The thickness of emerging dendrites can be estimated from the most unstable modes as

$$\xi \approx \{[\gamma/C'(R_{\min})]\partial V/\partial C_0[1 + (R_0/D)\partial j/\partial C_0]\}^{1/3}, \tag{10}$$

and is set by an interplay of membrane rigidity γ which tends to thicken the outgrowths and the increase in growth rate with morphogen concentration $\partial V(C_0)/\partial C_0 > 0$ which must be positive for cellular dendritic morphogenesis to occur, and also thins them. We also suggest that the ratio $R_{\min}/\xi$ gives an order of magnitude estimate of the maximum number of dendrites that the growing cell can elaborate.

A second mechanism follows from the fact that an initially constant morphogen concentration C_1 [the solution of $j(C_1) = 0$] can become unstable [13] to concentration fluctuations if $\partial j/\partial C_1 > 0$, and indeed the mode $\delta C(r,\theta,t) = c_m(t)[r/R_0(t)]^m\cos(m\theta)$ becomes unstable when $R_{0,m} = Dm/(\partial j/\partial C_1)$, resulting in endogenous morphogen currents. As a result of such spatial inhomogeneities dendritic growth results, $d\delta_m/dt = (1 - m^2)[\delta_m m^2\gamma/R_0^4 + c_m\partial V/\partial C_1]$. As dendritic growth first appears for $m = 2$, a similar form for the instability condition $R_{\min} = 2D/(\partial j/\partial C_1)$ is found for this case also.

For simulations, we discretize our equations on a lattice. This results in three types of lattice points: internal domain points, external domain points, and a set of contiguous points representing the membrane. A Jacobi relaxational procedure, together with the boundary conditions given by the sum of the diffusive and pumped fluxes, yields the equilibrium concentration at each internal lattice point. The membrane is treated as a discrete linked list of data structures, containing information on the local submembrane calcium concentration and therefore on the local growth velocity $V_i = V(C_i)$ at a point i on the neuronal surface. All sites with $V_i < 0$ are retracted. A stochastic growth rule is then employed at each time step, with the probability $P_i = V_i/V_{\max}$ that growth occurs. If one of these sites is not chosen to grow, then the probability of retraction is chosen so that on the average a constant global addition of material to the cell occurs. Whether growth or retraction actually does occur with these probabilities is constrained by the demands of topology, rigidity, and hard core repulsion—approach of processes closer that the diameter of a growth cone with filopodia was disallowed. If growth or retraction is allowed (no pinching off of membrane and resulting local curvature not too large), then the membrane lattice point in question is replaced by an internal (growth) or external (retraction) domain point, and any resulting break in membrane contiguity is mended.

Simulations of Eqs. (1)–(4) in voltage-gated vesicles can be seen in Fig. 1 for both $\alpha = 0$ (calcium independent growth) and $\alpha = 1$ (calcium catalyzed growth), starting from small circular domains with low internal calcium levels. Morphogen independent cellular growth gives rise to no dendritic arboring, the morphology being similar to Eden growth, and the internal calcium concentration remaining at low levels. For calcium catalyzed growth, however, a diffusive influx occurs at random protuber-

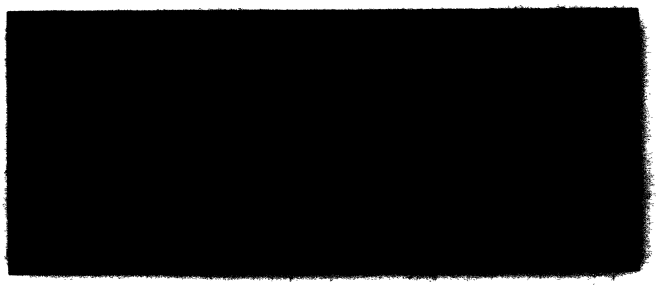

FIG. 1. The influence of the morphogen on cellular dendritic growth in growing voltage-gated vesicles. Growth has been rendered calcium independent $\alpha = 0$ in the left image, while calcium-dependent growth ($\alpha = 1$) is shown right.

ances which grow allowing further influx. When the internal calcium rises to $\approx C_{crit}$, active extrusion sets in. Quasistatically the morphogen concentration equilibrates into a spatially inhomogeneous form. As the diffusive influx of calcium is larger at the tips of dendrites than elsewhere because of their larger surface to volume ratio, a higher calcium concentration can be expected in the dendrites, and this is indeed observed in both the simulations (see Fig. 1) and experimentally in the large intracellular calcium gradients seen along growing neurites of mollusc neurons *in vitro* [8]. This higher concentration induces further cytoskeletal polymerization resulting in cellular dendritic growth.

In conclusion, note that for both mechanisms voltage-gated membranes are required, and in both cases the mechanisms are stabilized by internal morphogen diffusion. This is in direct contrast to the Mullins-Sekerka instability where external diffusion enhances the instability. We have focused in this paper on calcium ion modulated neuronal growth, but it should be kept in mind that the calcium ion have been implicated much more broadly in morphogenesis, including regulation of cellular viscoelastic properties [16]. Another influence on gross cellular morphology is osmotic pressure $\Delta p = p_{in} - p_{out}$. This can be seen in the variation of shape or cytotype exhibited by membrane vesicles such as red blood cells with osmotic pressure [17]: swelling into spherical forms as $\Delta p \rightarrow \infty$ and shrinking into forms resembling branched polymers as $\Delta p \rightarrow -\infty$. This class of morphological changes is distinct from the mechanisms of dendrite growth, though the cytoskeletal scaffolding will constrain the influence of osmotic pressure on form.

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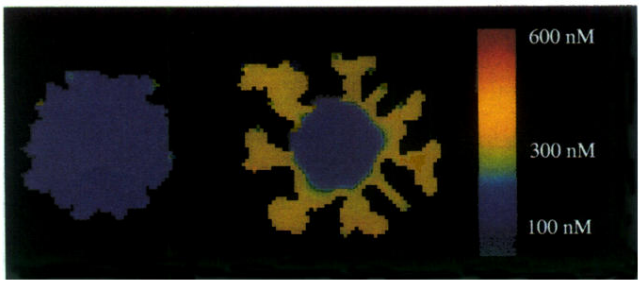

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