Moving toward molecular mechanisms for chemotaxis in eukaryotic cells

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ABSTRACT **It is a tremendous honor to receive the 2019 E.B. Wilson Award and be recognized for my work on chemotaxis in eukaryotic cells. In writing this essay, I hope to achieve three aims: 1) to tell the story of how people in my group made discoveries over the years; 2) to outline key principles we have learned about chemotaxis; and 3) to point to the most important outstanding questions.**

A CIRCUITOUS ROUTE TO GUIDED MIGRATION

I grew up in a loving household in West Long Branch, New Jersey. My parents, children of Greek and Italian immigrants, had moved from Brooklyn for my father's first job as a mechanical engineer. My mother was a homemaker. I had an early affinity for math, which I eagerly learned from my father. Throughout high school, I was involved in sports, especially as quarterback on the football team. Our 1963 undefeated team will be inducted into the Shore Regional Hall of Fame this October! I started college in Lafayette (then all men) and finished at Wisconsin Madison. Although I was intrigued by life science since high school biology class, I studied physics. In retrospect, it seems like I spent most of my time socializing, but I was accepted into the biophysics graduate program at Johns Hopkins. Then, like many students at the time, I took off for a summer of backpacking through Europe.

At the time, I never thought that I would spend my career at Johns Hopkins. The program in biophysics was rigorous, and my first real exposure to molecular biology was exciting. The neurophysiology classes by the late Martin Larabee left a lasting impression. I was extremely fortunate to land in the lab of Douglas Fambrough. We immediately clicked, and, with his guidance, I was able to transition from physicist to neurobiologist, making early contributions to acetylcholine receptor turnover. After interviewing for multiple postdoctoral positions in neurobiology, I heard about and was intrigued by the spontaneous cellular aggregation of *Dictyostelium*. Trying to leverage my expertise, I arranged to work with membrane biologist Theodore Steck, who was initiating collaborative studies with a *Dictyostelium* lab. Although my mentor was an excellent scientist, the other lab was in turmoil. Still, I managed to work out cell–cell signaling mechanisms and visualize the extracellular cAMP waves that organize the cellular aggregation. I also inherited several mutant strains that would later become important.

The successful PhD deserved another 3-month break. I signed on to an overland trip from London to Katmandu. Twenty-three of us from many countries, including one American and one French person, traveled in a British Army truck through Iran, Afghanistan, Pakistan, and India, camping out, eating local food, and having amazing experiences. The French woman was Aline Sanseau. Since I was the only one on the trip who spoke rudimentary French, we became friends. Three years later, she visited me in Chicago, and, to make a long story short, we just celebrated our 39th wedding anniversary.

THE FIRST CHEMOATTRACTANT RECEPTORS

I was fortunate that the new chair in Biological Chemistry at Johns Hopkins School of Medicine, Daniel Lane, who had just taken over from Al Lehninger, recognized my graduate and postdoctoral work and hired me in 1980. Senior members in the department, such as Dan, Paul Englund, and Bill Lennarz, and peers such as Don Cleveland and Gerry Hart, stressed rigorous biochemistry. The scientific world at this time was in a frenzy of purifying and cloning, with large teams identifying gated ion channels, receptors, and G-proteins.

Our small group focused on the cAMP chemoattractant receptors in *Dictyostelium*. Our only handle was a weak photoaffinity-labeled band, but graduate student Anne Theibert and research specialist Jane Borleis noticed that the band shifted during cAMP occupancy. Barry Knox and I spent time at the Weizmann Institute with the late Lee Segal and Alfred Goldberger to model the behavior. Characterization of this phosphorylation event by Anne,

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Barry, and Roxanne Vaugh gave us a much stronger assay for the protein. Over time, we harvested 4 kl of cells to obtain enough protein to raise an antibody. When Peter Klein, an exceptional MD–PhD student, and collaborators Karl Saxe and Alan Kimmel cloned cAR1, we saw that *chemoattractant receptors resembled the seven transmembrane structures of rhodopsin and yeast STE2*. The β-adrenergic receptor sequence was published just before ours, and the GPCR family of receptors emerged. Kimmel's lab, together with Julia Sun and Ron Johnson in my group, quickly discovered a family of cAMP receptors with different affinities and functions. An interesting note on the origin of terminology: James Bear in Karl Saxe's lab later went on to identify a suppressor of cAR2 deficiency which became the well-known actin regulator, SCAR (Bear *et al*., 1998).

This led us to identifying many of the obvious players in the cAMP cell–cell signaling module following the work from the Gilman and other groups on hormone signaling. In collaboration with Rick Firtel, Rob Gundersen, Maureen Brandon, Geoff Pitt, and Lijun Wu found and characterized eight G-protein α-subunits and showed that α2 was mutated in a series of mutants unable to respond to cAMP. Graduate students Pam Lilly and Ning Zhang cloned the β- and γ-subunits; it later became very useful that *Dictyostelium cells contain a single βγ-complex*. Finally, MD–PhD student Geoff Pitt cloned the adenylyl cyclases in collaboration with molecular biologist Randall Reed. Gene disruptions of all of these genes showed that receptors and G-proteins were essential for chemotaxis, but cAMP production was necessary only for cell–cell communication. Dale Herald, Michael Caterina, Ji-Yun Kim, Tian Jin, Jacqueline Milne, and Carole Parent used random mutagenesis to identify many residues critical for the function of the cARs, G-proteins, and adenylyl cyclases.

EARLY STUDIES OF CHEMOTAXIS

Chemotaxis in eukaryotic cells, found in simple organisms such as starfish larvae, was recognized as a universal phenomenon by E. Metchnikoff, who received the Nobel prize in 1908 (Metchnikoff, 1887). However, in the 1980s and 1990s few labs were studying it, and, outside of the immune system, it was not considered to play much of a role in development or physiology. At that time, I frequented the Sensory Transduction in Microorganisms Gordon Conference, which was intensely focused on bacterial chemotaxis. I was inspired by the beautiful work of Julius Adler, Mel Simon, Sandy Parkinson, and John Spudich presented there, and those studying prokaryotic chemotaxis were very encouraging to the few *Dictyostelium* investigators such as Jim Spudich, Günther Gerisch, and myself.

We, among others, set out to use *Dictyostelium* for a genetic analysis of chemotaxis. The cells were physiologically amenable but the genetics was difficult because fine-structure mapping, available in yeast and flies, was arduous. This was somewhat mitigated when Bill Loomis' group reported REMI, which allowed random insertional mutagenesis (Kuspa and Loomis, 1992). Many "chemotaxis" mutants were isolated by simply isolating clones that failed to aggregate. Characterization of these mutants by the Van Haastert, Firtel, and other groups and by Linnan Tang, Saskia Van Es, Stacey Willard, Carol Manahan, and later Yulia Artemenko and Tom Lampert led to a large interconnected network of signal transduction events triggered within seconds of chemoattractant exposure (Van Haastert and Devreotes, 2004; Janetopoulos and Firtel, 2008). As far as is known, a similar network of events is triggered by chemoattractants in human migratory cells such as neutrophils.

Three of the most important mutants from our group were *Aimless* isolated by Rob Insall, *Pianissimo* isolated by Mei-Yu Chen, and *Synag* 7, which I had brought from Chicago. All of these mutants were defective in cAMP production and chemotaxis. *Aimless* was a RasGEF, which probably gave the first indication that *Ras, in addtion to Rac, family proteins are important in chemotaxis*. We published *Pianissimo* as a highly conserved novel chemotaxis gene, also essential for yeast growth. *Pianissimo was later rediscovered as signature subunit, Avo3/Rictor, in yeast/mammalian TorC2 complexes* (Loewith *et al*., 2002).

I had earlier found that the inability of *Synag* 7 to produce cAMP could be reconstituted in vitro by addition of wild-type supernatants. Using this assay, graduate student Pam Lilly carried on a heroic 3-year purification of the reconstituting factor in the supernatant. Using sequence from a small amount of protein isolated by Pam and REMI mutants from the Loomis lab, we identified the cytosolic regulator of adenylyl cyclase, Crac, a PH-domain containing protein without outside homology. The requirement for a cytosolic protein in receptor-mediated activation of adenylyl cyclase was inconsistent with accepted models of this module (Gilman, 1995).

GETTING FROM CHEMOATTRACTANT RECEPTORS TO THE CYTOSKELETAL MACHINERY

During this period, studies of chemotaxis were more or less limited to observations of cells and identification of components. I was inspired by the observations of Günther Gerisch in directing cells with cAMP-filled micropipettes (Gerisch and Keller, 1981). I learned a great deal about the physiology of the process in neutrophils from endless discussions with Sally Zigmond. The similarities to *Dictyostelium* were remarkable, and the identification of the chemokine receptors as GPCRs strengthened that view at the molecular level. The advent of GFP opened up studies of cell biology, and chemotaxis was a major beneficiary. Zhan Xiao showed, to everyone's surprise, that *cAR1-GFP was uniformly distributed along the cell perimeter*. Similarly, the G-protein subunits were largely uniform.

This implied that downstream signal transduction events were needed to connect the external gradient to the localized cytoskeletal responses at the front and back of the cells. Visualization of the PH domain of Crac in living cells led to a major breakthrough: Unlike the receptors and G-proteins, *Crac was recruited from the cytosol to the leading edge of chemotaxing cells*. A series of experiments carried out by Carole Parent and Yi Elaine Huang showed that *this localization reflected an accumulation of PIP3*. This was one of the earliest biosensors for visualization of signal transduction events.

One of the most striking results was the ability of chemotactic gradients to localize PIP3 in completely immobilized cells. This showed that *chemotactic cells have a spatial sensing mechanism; they can sense slight differences in receptor occupancy across their length*. It also meant that *directional sensing and cell motility are separable*. Studies by others and by Min-Jie Wang later demonstrated that polarity was a third separable process. *Polarized cells respond to chemotactic gradients by vector addition of directional response and polarity vectors*.

The spatial–temporal responses of immobilized cells led us to propose that *receptor occupancy generates rapid, Local Excitatory and slower, Global Inhibitory processes (termed LEGI)*, inspired by models for adaptation to chemoattractants in bacteria. With uniform stimuli, responses start as excitation initially exceeds inhibition but, eventually, they subside as the processes balance. Further responses can be triggered by additional stimulus increments. With spatial stimuli, the local excitation remains persistently higher than the global inhibition at the front, whereas inhibition always exceeds

excitation at the back. Working with control engineer Pablo Iglesias, Lan Ma, and Lian Liu, Chris Janetopoulos obtained extensive experimental and computational evidence supporting the LEGI hypothesis. It retains its predictive value today, although the molecular nature of the inhibitor remains elusive. This also began a long and productive collaboration with Pablo.

The studies of PIP3 as a marker for the front of the cell opened tremendous possibilities. First, Miho Iijima in the lab identified the PIP3 phosphatase PTEN and showed that it localized to the rear of the cell, opposite to PI3K (as had been shown by the Firtel lab). The complementary localization of PI3K and PTEN ensured a steep accumulation of PIP3 at the front. It also led to the idea that *domains on the plasma membrane are dynamically separated into "front" and "back" states*. This concept is fundamental to our understanding of cell migration today. Second, it clearly showed the relevance of *Dictyostelium* as a model for mammalian cells. Human tumor suppressor PTEN was characterized using *Dictyostelium* by Miho, Paquita Vazquez, Meghdad Rahdar, and Nghia Nguyen. PIP3 was quickly found at the leading edge of neutrophils and fibroblasts, and, by now, this principle has been demonstrated in many other cells and polarity situations.

Overproduction of PIP3 caused a striking phenotype, but severe reduction had only a weak effect that was context dependent, suggesting that there was redundancy in the pathway. Yoichiro Kamimura traced part of the redundancy to a homologue of protein kinase AKT, which shared almost all of its substrates. While AKT required PIP3 for activation, this second kinase did not; it needed only TorC2 for activation. Another source of redundancy was found with Lingfeng Chen's discovery that PLA2 functionally synergized with PI3K. In an attempt to find more essential components, Huaqing Cai and Yu Long disrupted and overexpressed constitutively active Ras family members. The Weeks and Firtel labs had previously demonstrated that multiple Ras proteins were activated by chemoattractants (Lim *et al.*, 2002). Huaqing found one Ras directly activated TorC2 and that the phenotypes produced by activation, compared with deletion, of Ras proteins were much stronger. This again suggests that components in the signal transduction network are highly interconnected and redundant.

CHEMOATTRACTANTS BIAS SELF-ORGANIZING ACTIVITY IN THE SIGNAL TRANSDUCTION NETWORK

A series of observations began to chip away at the textbook view of chemotaxis that cytoskeletal events drive random motility while signal transduction events merely connect receptor occupancy to the cytoskeleton for guidance. First, as cells unable to receive external stimuli due to deletion of the G-protein βγ-complex moved about randomly, signal transduction events such as Ras and PI3K activation nevertheless appeared at the tips of protrusions. Second, in writing a review, Kristen Swaney and I realized that besides receptors and G-proteins, there were few signal transduction components specifically required for directional sensing and not motility. Third, a screen for electrotaxis mutants by collaborator Min Zhao's student Ranchi Gao yielded the motility mutants we had already identified from chemotaxis studies.

A series of observations came together to suggest that our initial views of chemotaxis were quite naïve and the process was more amazing than we had imagined. These included 1) Günther Gerisch's characterization of actin waves discovered by Michael Vicker in *Dictyostelium* and Orion Weiner and Mark Kirschner's observation of actin waves in neutrophils (Vicker, 2000; Weiner *et al.*, 2007). 2) Hans Meinhardt's application of his reaction diffusion models for development to dynamic polarity and Tobias Meyer's simulation that a slight bias of spontaneous protrusions could bring about guidance (Meinhardt, 2000; Arrieumerlou and Meyer, 2005). 3) Masahiro Ueda's demonstration of beautiful spinning waves of PIP3 and PTEN in rounded latrunculin-treated *Dictyostelium* cells (Arai *et al.*, 2010). 4) Bear Huang and Michelle Tang's observation of spontaneous traveling waves Ras and PI3K activation coupled to actin waves in migrating cells. On the basis of these results we envisioned an excitable network hypothesis: The signal transduction network transiently shifts between "back" and "front" states in domains of the plasma membrane that activate sequentially like a moving "stadium wave." Front state biosensors such as Ras-binding domains are recruited, whereas back state biosensors such as PTEN dissociate, appearing as complementary "shadow" waves. We proposed that *a signal transduction excitable network (STEN) is necessary to organize a cytoskeletal excitable network (CEN) to generate the large protrusions that mediate cell movement*. Thus, with the exception of G-proteins, all the events previously believed to only connect receptor occupancy to the motility are actually part of motility.

Yuan Xiong in Pablo Iglesias' lab and Bear developed an elegant application of the Fitzhugh–Nagamo equations for excitable systems to explain spontaneous activity and the propagating waves. They coupled the excitable system to the LEGI module to explain directional sensing and suppression at the back. This biased

FIGURE 1: Devreotes lab barbeque 2014, including many alumni and families.

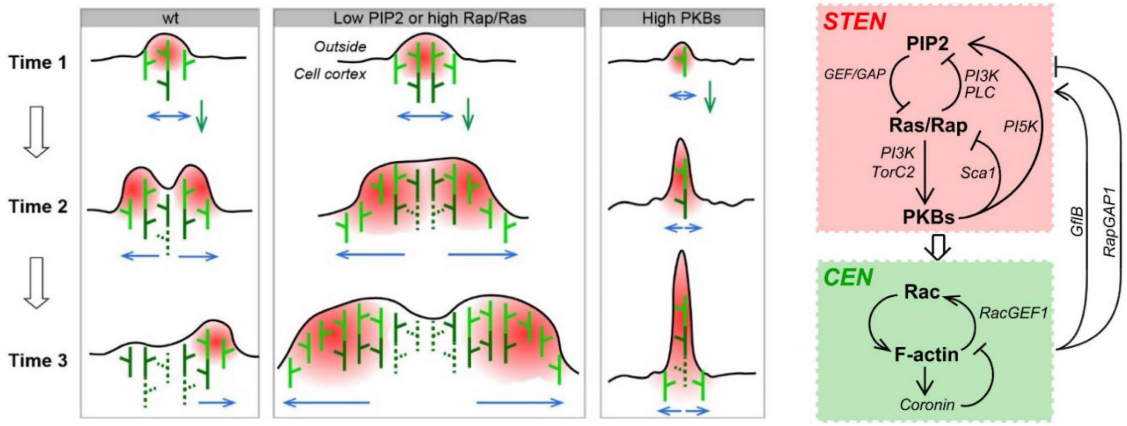

FIGURE 2: Left, range of lateral travel of waves of signal transduction activity (red) that determine the size of protrusions mediated by orthogonal actin polymerization (green). Right, schematic view of feedback loops that underlie excitability of the signal transduction and cytoskeletal networks. From Miao *et al.* (2019).

excitable network (LEGI-BEN) model was quite similar to the Meinhardt model, although LEGI-BEN accounted for adaptation—the ability of cells to sense gradient steepness regardless of the absolute concentration. Simulations by Pablo's students Changji Shi and Sayak Bhattacharya showed that these models could account for most of the temporal and spatial responses to chemoattractants. *The models predicted that slight changes in the strength of feedback loops could lead to extremely different cellular behaviors such as oscillations and constitutively activated cells.*

EXCITABLE NETWORKS THAT DEFORM THE CELL CORTEX

Feedback loops are easy to incorporate into equations, but I was perplexed about how to approach the molecular counterparts. The chemically induced dimerization systems that Takanari Inoue had been developing provided a way to suddenly activate a "downstream" point in a network and learn its effect "upstream" as would be expected for feedback (Suh *et al.*, 2006). Takanari and I comentored Yuchuan Miao, who discovered that small perturbations at multiple single nodes could shift the state of the entire network. Lowering the threshold for excitability, as predicted by the models, could switch cell behavior from amoeboid to keratinocyte-like to oscillatory. As shown in Figure 2, lowering threshold increases the range of traveling waves, which determine the lateral dimensions of protrusions. *These studies demonstrated that cellular morphology and migratory mode are fluid.* We proposed that the varied protrusions made by cells, such as lammelipodia, pseudopodia, filopodia, macropincytotic cups, podosomes, and invadiapodia, are on a spectrum, and one can rapidly transition between them by small shifts in a signal transduction network that controls the cytoskeletal activity.

Our current studies are focused on delineation of the feedback loops in the network that bring about the excitability shown in Figure 2. Several recent insights suggest how positive feedback may come about. Kristen Swaney found a novel "back" protein that binds to PI(3,4)P2, and Xiaoguang Li and Marc Edwards found that, while *activations of Ras drive PI(3,4)P2 levels down, lowering of PI(3,4)P2 increases Ras activity. Similarly, lowering of PI(4,5)P2 also leads to activation of Ras.* These mutual inhibitory interactions comprise important positive feedbacks. Current thoughts on delayed negative loops involve activation of PKBs by PIP3 and TorC2. PKB-mediated phosphorylation of Ras Gef Aimless is thought to mitigate Ras activation while phosphorylation of PI5K activates the enzyme to elevate PI(4,5)P2, further inhibiting Ras.

KEY QUESTIONS FOR FUTURE STUDIES OF CHEMOTAXIS

The fact that so many signal transduction activities are coordinately regulated suggests that there is an overarching organizing physical property. The existence of this property is an important question for future research. We are currently working on the idea that this organizing activity is surface charge on the inner leaflet of the plasma membrane. Electrical excitability in neurons derives from the fact that channels controlling membrane potential are themselves voltage dependent. I believe that the biochemical excitability underlying cell motility may derive from the ability of the enzymes controlling a physical property of the membrane are themselves regulated by that property. For example, a shift in surface charge could differentially recruit activities, which in turn ultimately regulate the surface charge.

One of the most important questions is the extent to which these concepts are conserved across eukaryotic cells. The similarities among chemotactic cells in simple organisms and humans observed by Metchnikoff more than a century ago started to be established at the molecular level by comparison chemoattractant receptors and G-proteins in *Dictyostelium* and neutrophils. As additional features of chemotactic systems have been uncovered, the parallels have been extended. Recently, we have observed traveling waves of signal transduction activity remarkably similar to those in *Dictyostelium* in human neutrophils and epithelial cells. Thus, the molecular mechanisms controlling critical physiological events such as inflammation and wound healing and pathologies such as cancer metastasis can be traced to their origins in simple eukaryotic cells.

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