HISTORY OF NEUROSCIENCE: DAVID J. ANDERSON

1. Origins

In retrospect, I had no business becoming a biologist. As a child and adolescent, I showed none of the phenotypic characteristics that identify a budding young scientist: I didn't collect bugs and burn them on the sidewalk with a magnifying glass; didn't take radios apart and put them back together; had no aptitude for math, physics, computer programming or chess; didn't like reading fantasy and science fiction and avoided trying to solve complicated puzzles because I was easily frustrated. Instead, I enjoyed writing short stories; photography and filmmaking; listening to and playing acoustic guitar; acting in plays; writing musical parodies; learning foreign languages and collecting hats. Clearly, I had inherited most of my dominant genetic predispositions from my mother Helene (Masslo) Anderson, a Professor of Spanish Literature at NYU, and few if any from my father James L. Anderson, a mathematical physicist specializing in General Relativity who was a Professor at Stevens Institute of Technology in Hoboken, NJ. They met and married while in graduate school at Syracuse University, and then had me as their first child in May of 1956. I guess if you cross a physicist with a humanist, you get a biologist.

In the summers, my parents couldn't afford to send me to sleepaway camp. Instead, they brought me every summer to Woods Hole, MA where my father taught in a yearly WHOI (Woods Hole Oceanographic Institute) course in geophysical fluid dynamics (why that has anything to do with black holes and Einstein's Theory of General Relativity I never understood and probably never will). Because of WHOI I briefly considered becoming an oceanographer; they looked manly, with their thick beards and flannel shirts, and went to sea for months at a time like 19th-century whalers. But down the street from WHOI was the MBL (Marine Biological Laboratory), where 99% of the scientists who flocked to Woods Hole each summer had laboratories and did research while their children attended Science School and frolicked on the sands of Stony Beach. All my summer friends were the children of biologists like George Wald and Jerry Hurwitz. They had summer jobs in their fathers' labs, while I stocked tampons in the basement of the Woods Hole Pharmacy. It made me feel like the proverbial kid with his nose pressed up against the glass window of a candy store. They seemed to be having a lot more fun at their jobs than I did, and I desperately wanted in. However, it was virtually impossible for a kid who wasn't the child of an MBL scientist to get a summer job in a lab there.

Fortunately, a biologist friend of my parents named Robin Wallace, who worked at the Oak Ridge National Lab during the winter and in the summer at the MBL, and whose own boys weren't interested in a lab job, came to the rescue and got me a job washing test tubes for the graduate students in FERGAP, the Fertilization and Gamete Physiology Program. It was menial labor, but I was *in*! I soon became enamored of the easy camaraderie among the students, and their sense of humor. Although I was just entering 10th grade, I started attending FERGAP lectures and particularly enjoyed a special lecture by Joel Rosenbaum, a Yale Professor, on the Synthesis of Microtubules In Vitro. I barely knew what a microtubule was, but the fact that you could take them apart and then watch them spontaneously reassemble in a petri dish fired my imagination and kindled an interest in Cell Biology.

I started reading papers in the MBL Library and became fascinated with the cadence and rhythm of scientific writing. Having inherited my mother's ear for languages and talent for mimicry, as well as a love of writing, I soon found myself trying to imitate the literary style of a scientific research paper. Arriving in the lab each morning to see all the test tubes I had studiously washed the previous day piled up in a basin next to the sink following the graduate students' nocturnal experiments, I was inspired to write a parody of a microtubule assembly paper, called "*Synthesis of Glassware In Vitro*". Complete with figures and graphs, it described how subunits of "tubeulin" could spontaneously self-assemble overnight into conical glass centrifuge tubes in a plastic bucket, in the presence of some salt, buffer and ATP. I described how accidently subjecting such assembled tubes to the Earth's gravitational field caused rapid acceleration followed by an instantaneous and irreversible increase in entropy. Together with a fellow dishwasher, Paula Grant (the daughter of our biologist neighbor Philip Grant), we typed up the paper and presented it to the graduate students in the course. It was an instant hit and everyone wanted copies. I'd never experienced this kind of reception for anything I did, and I was immediately hooked. What I failed to grasp was the lesson that I should have become a comedian or satirist, rather than a scientist.

2. Harvard college: falling in love with cell membranes

My summer lab experience prompted me to take Daniel Branton's course in Cell Biology during my first term at Harvard. I had wanted to go to Yale to work with Joel Rosenbaum on microtubules but was (of course) waitlisted so I chose Harvard, which was clearly an institution with lower academic standards. Branton was a fabulous lecturer. While I enjoyed the course overall, what really captured my imagination was his lecture on the new "fluid mosaic model" of cell membranes, proposed by S.J. Singer and John Nicholson[1]. For some reason, I found the concept of protein "icebergs" floating in a lipid "sea" to be fascinating. I asked Branton if I could do research in his lab, where they were testing the concept of lateral diffusion of integral membrane proteins using the erythrocyte membrane as a model system, and fortunately, he took me on. While juggling experiments and rehearsals for theatrical productions didn't allow me to get much done in the lab at first, I learned a valuable lesson: if you want to make progress in science you have to focus on one problem and not try to do too many things at the same time. This was (and still is) challenging for me because I find so many things interesting that I have difficulty deciding which problem to choose, but learning the discipline to focus on one project, even if I wasn't sure I made the right choice, was an important lesson.

In the Spring term of my junior year (1977) I took Bio 25, Introduction to Neurobiology, which was taught by John Dowling (see Volume 4) with guest lectures by Torsten Wiesel and others from the Harvard Medical School Department of Neurobiology. Torsten at the time still had a fairly strong Swedish accent, and at first I couldn't understand why he kept talking about "urinals," as in the "Urinal of Physiology" or the "Urinal of Comparative Neurology". It made me wonder whether I was using the wrong bathroom. The course used the classic textbook *From Neuron to Brain* by Steven Kuffler and John Nicholls. I found the course interesting from the perspective of biological membranes and their biophysical properties, but the importance of orientation and ocular dominance columns was lost on me.

I wound up doing my senior honors thesis in Biochemical Sciences (my major, or "concentration" as they called it at Harvard) in the Branton lab, but my most interesting discovery—that cationic artificial lipid vesicles fused with erythrocyte membranes were maintained as segregated lipid rafts, into which endogenous membrane proteins could not diffuse—turned out to be an artifact (how I managed to graduate Summa I'll never know). It was a crushing blow to discover that all the results I had worked so hard to obtain were essentially meaningless. I don't remember why it was artifactual or how I discovered it, but I do remember Dan Branton saying to me "I hope something like this never happens to you again." Unfortunately, I mis-interpreted his suggestion as encouragement to forge ahead in science despite such setbacks, rather than as advice that maybe I should choose another line of work.

3. Rockefeller: synthesis and membrane integration of acetylcholine receptor subunits in vitro

My senior thesis research on the lateral diffusion of integral membrane proteins led me to wonder how cells were able to maintain segregated domains of such proteins in discrete regions of the cell membrane, such as acetylcholine receptors at the neuromuscular junction. My time in Dan Branton's lab had coincided with the exciting discovery there, by the talented biochemist Van Bennett, of spectrins and ankyrins, proteins that bridged erythrocyte integral membrane proteins with the underlying cytoskeleton and restrained them from lateral diffusion in the plane of the membrane. Later, isoforms of these proteins would prove to play a pivotal role in neurons in maintaining isolated domains of membrane protein function, such as clusters of voltage-gated sodium channels at Nodes of Ranvier. But instead of trying to extend Bennet's work to neurons (which would have been the smart thing to do), for graduate school I decided instead to study what I thought was the most extreme example of such segregated membrane protein domains: the gap junction. To this end, I wrote an NSF graduate fellowship proposal to work with Norton Bernard Gilula (a former postdoc of Dan Branton's) at The Rockefeller University. This decision was in part driven by the fact that Bernie Gilula studied gap junctions using freeze-fracture electron microscopy, a technique I had used extensively in my undergraduate research with Branton, and that gave me a comforting sense of familiarity and continuity with my work at Harvard.

I got my NSF fellowship and was accepted to Rockefeller (which paid me the princely stipend of $5,000 per year instead of the paltry $3,500 offered by NSF), and in the Fall of 1978 eagerly began work in Bernie Gilula's lab to isolate biochemically gap junctions and characterize their molecular composition (this was long before the connexins were discovered). However, I soon found that this was much more difficult than I had anticipated, for several reasons. First, gap junctions were present in extremely low quantities even in the liver, their most abundant source. On my first day in Bernie's lab we guillotined 200 rats and purified their gap junctions on sucrose gradients; our yield was a paltry few hundred micrograms of protein. Second, there was no enzymatic or bioassay for purified gap junctions at the time, other than looking under the electron microscope. Therefore, one could not track protein enrichment and activity, as in a traditional biochemical purification scheme. In theory, a purified sample could be highly enriched for gap junctions seen in the EM and show a couple of abundant bands on an SDS polyacrylamide gel; but the prominent bands might represent an invisible contaminant rather than the visible isolated gap junctions. At one point we tried to solve the problem by switching to bovine lenses (which were then thought to be highly enriched in gap junctions) as a source of

material but ran into the same problem of not having an assay. In retrospect that was fortunate, because the bovine lens protein I was studying, MP26, later turned out to be an aquaporin (water channel) rather than a *bona fide* gap junction protein. On top of that, Bernie was not a biochemist by training, but rather an electron microscopist who was unable to provide the kind of expert advice in protein purification that I needed to make progress. So reluctantly (and to Bernie's great disappointment) I decided to switch labs.

The question was, to which lab? Because I had been so myopically focused on gap junctions and Bernie Gilula, I hadn't bothered to pay much attention to the other Rockefeller faculty and their research. So at first I felt completely unmoored and lost. Eventually, however, I found a new lab – which I chose for completely non-scientific reasons. I had become friendly with Peter Walter, a second-year student from West Berlin with a penchant for practical jokes who looked like Santa Claus with a brown beard. Peter was working in a spanking brand-new lab that had recently been renovated for his adviser, Günter Blobel. Blobel was then a 35 year-old German immigrant who had been promoted to Assistant Professor after doing a postdoc at Rockefeller with Nobel Laureate George Palade. I had barely heard of Blobel – his famous "signal hypothesis," for which he ultimately won his own Nobel Prize, was not yet published when I took Cell Biology in 1974. But it seemed to be a very exciting time in the Blobel lab, and there was a nice empty bench opposite Peter's where I could at least work nearby my new friend. So, I tried to think of a PhD project that I could do uniquely in the Blobel lab, that would interest Günter and be relevant to my budding interest in neurobiology. But truly this was just a pretext to join his lab so I could escape from Bernie's and engage in practical joke warfare with Peter Walter.

The question was, what project could I do in Günter's lab that had any relevance to neurobiology? At the time, Günter and his postdoc Bernhardt Dobberstein had already done their ground-breaking experiment demonstrating that supplementing an in vitro translation system (prepared from yeast or a reticulocyte lysate) with canine pancreatic rough microsomal membranes--vesicles derived from the rough endoplamic reticulum, or ER--was sufficient to reconstitute the co-translational translocation of a secretory protein across the microsomal lipid bilayer and into the ER lumen with concomitant signal peptide cleavage[2]. This experiment was the most compelling evidence for the signal hypothesis, originally proposed by Blobel and David Sabatini, which posited that ribosomes translating mRNAs for secretory proteins were targeted to the ER not by signals in the mRNA, but by short amino-terminal extensions of the nascent polypeptide chain they called "signal peptides". At the time I approached Blobel about transferring to his lab, he was focused on generalizing his discovery to other types of secreted or membrane proteins, as well as to other organelles such as lysosomes and mitochondria. There was already evidence that a transmembrane protein could hitchhike onto the same microsomal translocation system that worked for peptide hormones (such as pre-prolactin). However, that membrane protein was a simple, single-pass transmembrane polypeptide from a virus.

I thought that Günter might be interested in testing in his system a non-viral, multi-pass integral membrane protein (one whose polypeptide chain snaked back and forth across the lipid bilayer several times instead of just once), and which was part of a multi-subunit protein complex, and indeed he was. The one that fit the bill, and had relevance to neurobiology, was the acetylcholine receptor (AChR). The AChR had been purified from abundant sources such as

the electric organ of electric rays and eels, but there was dispute about its subunit composition. Jean-Pierre Changeux in Paris had initially argued that it consisted of a single subunit (alpha), but more compelling evidence from Arthur Karlin and colleagues at Columbia suggested that there were four subunits, α, β, γ and δ, present in a 2:1:1:1 stoichiometry. I suggested to Günter that I purify the receptor from electric ray, prepare antibodies to each of its subunits and program his microsome-supplemented in vitro translation system with mRNA from the same electric tissue. I could use the antibodies to immunoprecipitate each of the four subunits and test whether they were properly integrated into the membrane. If this worked, I could use the system to find out whether each subunit was translated separately from its own mRNA, or whether they were initially translated together as a polyprotein precursor and then cleaved post-translationally into the different subunits. (Although the latter mechanism was unlikely, it was still a formal possibility that could be decisively ruled out using this system.) Finally, and most importantly in the long run, the ability to identify and translate AChR mRNAs in vitro would provide a means to molecularly clone the genes. I gave Günter a stack of papers to read about the AChR. The following morning, I went into his office and he said "David, the acetylcholine receptor is the nirvana of integral membrane proteins!" I was in.

This was clearly a very ambitious project (particularly the cloning part), especially for a single beginning graduate student. But I was possessed of youthful arrogance, ignorance and ambition, and at the time the in vitro approach developed in Günter's lab provided a unique inroad to the problem and was low-hanging fruit. So, in some sense the choice of the project was driven by opportunism as well as by satisfying my continuing interest in studying the biogenesis and assembly of integral membrane proteins. From Günter's perspective, it raised several general ("fundamental") questions about the rough ER translocation system and its role in membrane protein synthesis that had not yet been addressed. So he gave the project the green light. I was admitted to his lab and assigned an empty, brand-new bench across from Peter Walter, just as I'd hoped.

In for a big shock

If you'd told me that as a graduate student, I'd be standing over a stainless steel sink wrestling with a huge, thrashing live electric ray that barely fit in the sink, wearing rubber gloves to prevent electrocution and trying to kill it by driving a screwdriver into its brain with a hammer, I would never have believed you. But that's exactly how I found myself in the fall of 1979. I had managed to make my own antibodies to each of the 4 subunits of the nicotinic AChR by purifying the protein from the electric organ of *Torpedo californica* using affinity chromatography on cobratoxin-Sepharose, cutting each subunit band out of a preparative SDS gel, eluting the protein from the gel and injecting it (with Peter's help over the summer while I was in Woods Hole taking the MBL Neurobiology course) into rats. Getting material to purify the receptor holoprotein turned out to be surprisingly easy: I ordered big chunks of frozen electroplax tissue from Pacific Biomarine in Venice, CA, smashed it up with a hammer, threw it in a Waring blender with some non-denaturing detergent and poured the extract over the affinity column, which I eluted with SDS prior to electrophoresis. The antibodies worked well.

Next, I needed to isolate RNA containing AChR mRNA(s), for in vitro translation. Naturally I assumed that the tissue with the highest amount of AChR mRNA would be the tissue with the

highest amount of receptor protein, so I just ordered more frozen *Torpedo* electric tissue and isolated RNA from it using the lab's standard protocol. But when I measured the yield by spectrophotometry it was shockingly low; a few tens of micrograms from close to half a kg of frozen tissue. By contrast, other people in the lab routinely isolated rat liver RNA in milligram quantities from a few hundred grams of fresh tissue. While one prep of rat liver RNA could provide enough for close to 6 months of experiments, the amount I isolated from *Torpedo* was barely enough for a few in vitro translation reactions. With this tiny yield I'd be running RNA preps twice a month instead of twice a year. Something had to be wrong.

As a beginner, naturally I assumed the problem was my own incompetence. But when I ran the standard lab protocol to isolate rat liver RNA, I got close to the expected yield. So I wasn't completely incompetent. I thought maybe there was a problem applying that protocol to the *Torpedo* electric tissue. RNA is notoriously unstable and subject to degradation during the purification process. I tried improved protocols suggested by local experts on mRNA isolation, but the RNA yields per gram of frozen tissue were no better.

The only other explanation I could think of was that the RNA got degraded during the process of freezing the tissue in Venice CA, over which I had no control. In desperation, I ordered a live *Torpedo californica* from Pacific Biomarine. Peter kindly drove me to the JFK cargo facility to retrieve a gigantic styrofoam box containing said living electric ray sloshing around in some seawater (at that time I didn't yet have a driver's license since I had lived in Cambridge and New York where you didn't need a car). We raced back to Rockefeller, opened the box and dumped the writhing monster into the sink where I proceeded to try to kill it – much to the hilarity of onlookers in the lab (who were apparently unconcerned by the prospect of my electrocution by 20 Amps and 50 Volts of current from the beast). Eventually the creature succumbed, and I did my best to dissect out the mass of electroplax tissue as quickly as possible and grind it up in the Waring blender with my super-duper RNA extraction buffer. During that process I noticed that the live electroplax tissue had the jiggly consistency of a jellyfish and realized that it consisted primarily of a stack of watery bags surrounded by a sheath of connective tissue with synapses between them – a primordial battery. That already identified one problem: the mass of actual RNA-containing tissue relative to water was vastly lower than in the case of rat liver, which is packed with cells full of RNA. So ~99% of the tissue I was extracting was water.

Now well versed in all the nuances of RNA isolation and having added multiple extra purification steps, I raced through the procedure as quickly as possible to ensure that the isolated RNA would remain intact. Heart beating with anticipation, I awaited the final isopropanol precipitation step expecting finally to recover a big white pellet of pure RNA, like I got from rat liver. But no dice: the yield was a bit better, and the RNA was translated more efficiently than my RNA from frozen tissue, but it was still an incremental increase and not the ten-fold improvement I was hoping that my rayicidal procedure would produce.

Crestfallen and depressed, I began to wonder how the electroplax tissue could have so much AChR protein and such a tiny amount of AChR mRNA. Then I recalled a talk I had heard by Miriam (Mika) Salpeter from Cornell, who was an expert on studying the dynamics of AChR turnover at the vertebrate neuromuscular junction (NMJ). Using a clever pulse-chase technique

that labeled the receptors with radioactive α-bungarotoxin followed by quantitative autoradiography, she found that the receptors, which were crowded together at ~20,000/μm^2 under the synapse, have a half-life of over 2 weeks! That's a relative eternity, considering that the typical half-life of a single-subunit, freely diffusing membrane protein is on the order of 17-20 hrs. I finally dawned on me that perhaps most of the synthesis of AChRs occurred during *Torpedo* embryonic or juvenile development, and that since the receptors were so stable once synthesized, in the adult the electroplax cells turned their mRNA synthesis way down.

I didn't know (and still don't know) if that hypothesis is correct. But it made me realize that perhaps the low yield of RNA from adult *Torpedo* electroplax tissue was not due to my incompetence (although I cannot formally exclude that since I never asked anyone else in the lab to try the procedure). Since I couldn't find a ready source of juvenile *Torpedo*, I resigned myself to the fact that I was going to have to make frequent RNA preps to continue with my project. The good news was that I could go back to using frozen tissue and not have to kill any more giant thrashing electric rays with my own two hands in the laboratory sink.

Crushed like a cockroach

Once I was able to produce high-quality, intact RNA from the *Torpedo* electroplax tissue, the rest of the project progressed in short order. Using a fancy experiment incorporating ^{35}S-N-formyl methionyl tRNA I was able to show that the subunits were synthesized from separate mRNAs, rather than as a polyprotein that was post-translationally cleaved to 4 different polypeptide chains. I also found that supplementation of the in vitro translation system with dog pancreas microsomal membranes yielded membrane insertion with signal peptide cleavage and core glycosylation of each of the 4 subunits (α, β, γ and δ). While from the standpoint of fundamental cell biology this was not too surprising (as noted by one of the reviewers who summarily rejected our paper from *Cell*), it was an important step towards the evolving science of so-called "Molecular Neurobiology". *Nature* rejected the paper, citing their recent rejection of a "very similar paper" (describing the cell-free synthesis of the four AChR subunits but without the proof of independent synthesis or membrane insertion as I had done)[3]. My paper was eventually published in *PNAS*, communicated by Jerard Hurwitz (my lab partner in the 1979 MBL Neurobiology course) on April 20, 1981[4]. Günter called this "publishing mafia-style."

At that point, having learned that no new fundamental cell biological principles were to be revealed by my experiments, Günter understandably lost interest in the project. To his surprise, however, he started getting lots of invitations to neurobiology conferences to talk about this work. Apparently, the paper had made a bit of a splash in the neuroscience community, perhaps not because of what it reported but rather because of what it portended: the eventual cloning and sequencing of the AChR and other important membrane excitability molecules through the application of then-nascent recombinant DNA techniques. Rather than embracing the attention, however, Günter dismissed this enthusiasm as simply evidence of neurobiologists' ignorance of the fundamental cell biological principles he had discovered 6 years earlier. ("Neurobiology," he once said to me, "ach, that's just 'Applied Cell Biology'. One of these days, you have to do something *fundamental*; it sharpens your thinking!") Fortunately for me, however, he asked the conference organizers if I could present the work in his place. Surprisingly most of them agreed, and suddenly I was jetting to Gordon Conferences, meetings at Cold

Spring Harbor and castles in the Loire Valley, and even a seminar at the famed Department of Neurobiology at Harvard. For someone part way through graduate school this was very exciting, and eventually led to my being offered several faculty jobs before even receiving my PhD (all of which I turned down because I felt I wasn't ready).

I would perhaps have merited the undeserved attention I was receiving had I accomplished my original (and most ambitious) objective of cloning the genes encoding the 4 AChR subunits. Even Günter felt this would 'put me on the map'. "You *have* to have those clones," he once told me, "they are like nuclear weapons; without them you don't get invited to the negotiating table!" But what did cloning the genes have to do with in vitro translation of the mRNAs? At the time there was no genome sequence, and techniques for cloning genes were in their infancy. The most direct approach was to screen DNA (or cDNA) libraries with radiolabeled nucleotide probes containing partial DNA sequences of the gene(s) of interest. But that sequence had to first be identified by obtaining at least a partial *protein* sequence for the polypeptide encoded by the gene, reverse-translating it to deduce its genetic code and then chemically synthesizing a DNA probe containing the corresponding nucleotide sequence. The AChR protein had not yet been fully sequenced (partial N-terminal sequence data was provided in 1980 by Michael Raftery, Norman Davidson and Leroy Hood at Caltech)[5], and the Blobel lab had as yet no experience in DNA synthesis. In the absence of screening libraries using a specific probe, the only other viable approach would have been to use what was then called "hybrid-selected" (or "hybrid-arrested") cell-free translation. In this approach, clones from a cDNA library were tested for their ability to hybridize to the mRNAs of interest (by forming DNA:RNA heteroduplexes), and these hybrids assayed for their ability to select the mRNA of interest (or alternatively to block its synthesis) using an in vitro translation system and immunoprecipitation with specific antibodies – exactly the system my experiments had established.

While I could have (and maybe should have) attempted this latter approach, I was put off by several considerations that made it a daunting prospect for a solo graduate student. First, I knew nothing about recombinant DNA techniques, including constructing and screening cDNA libraries (and neither did anyone else in Günter's lab), which were relatively recent developments and technically demanding. Second, the in vitro translation screening required large amounts of RNA containing the mRNAs of interest, which (as described in gory detail above) I was unable to obtain from *Torpedo* electric organ. Third, the project would require cloning not just one gene, but four. Finally, and perhaps most importantly, Shosaku Numa in Kyoto had begun to publish papers describing the cloning and sequencing of genes encoding the major neuronal membrane excitability molecules, including the voltage-gated sodium and calcium channels and the Na^+-K+ ATPase. He had a very large and efficient team working as an assembly line to clone such genes using synthetic DNA probes inferred from partial protein sequence to screen cDNA libraries they had prepared from *Torpedo* mRNA. It didn't take a genius to guess that his next target was likely to be the AChR.

While I was weighing this decision, in perhaps one of his most famous quotes Günter once strode into my lab while I was toiling at the bench to speak to a Japanese postdoc (Katsuyoshi Mihara) who was working furiously to establish an in vitro system to study post-translational import into mitochondria of nuclear genome-encoded genes. As the only person in the lab studying mitochondrial import, he was making slow progress and was getting a bit discouraged.

Moreover, he was facing stiff competition from Gottfried Schatz in Switzerland, who had an entire lab of 20 people working on the project. "Katsuyoshi," Günter intoned with his Teutonic accent, "*you are one, and they [Schatz's lab] are many. You have to work day and night, or you will be crushed like a cockroach!*" (This was his idea of giving someone a pep talk.) Years later when I visited Ira Mellman, a former postdoc at Rockefeller who had moved to Genentech, he had hanging on his wall a framed, traditional American-style needlework "sampler" made by his wife with Günter's quote in the center and cockroaches crawling around the edge of the frame.

With Numa's army of postdocs on the horizon marching towards cloning the AChR subunit genes, it was pretty clear that with my lack of technical expertise, even if I *did* work day and night I would be crushed like a roach. Indeed, using the partial protein sequence published by Raftery et al in 1980, Numa succeeded and published in *Nature* the cloning of all four AChR subunits in 1983[6, 7], only two years after I had published my in vitro translation paper. In the same year, a team from Steve Heinemann's group at the Salk Institute led by Toni Claudio also succeeded, using hybrid-selected cell-free translation, but managed to clone only the γ subunit[8]. Numa (who passed away in 1992 at the age of 63) and his team's brilliant work truly broke open the era of Molecular Neurobiology, and suddenly everyone wanted to follow suit. I decided that if I wanted to continue in this nascent field, I needed to acquire molecular biology expertise, and with an introduction from Eric Kandel (see Volume 9) wound up as a postdoc in Richard Axel's laboratory at Columbia's College of Physicians and Surgeons early in 1983.

Columbia: Out of the frying pan and into the fire

After the orderly, hierarchical structure of Herr Professor Doctor Doctor Blobel's Max Planck Institute-style laboratory at Rockefeller, moving to Columbia was a baptism-by-fire. The Axel lab had a chaotic atmosphere, with everyone working on completely unrelated projects, and was full of colorful personalities who frequently clashed. The contrast in styles between Blobel and Axel couldn't have been more dramatic. Günter was committed to realizing his vision of a unified model for what he called "Intracellular Protein Topogenesis[9]," a theory that explained the sorting and trafficking of proteins to various organelles (and for which he eventually won his long-sought-after Nobel Prize). In contrast Richard was a brilliant, eclectic opportunist who loved to jump into fields he had never worked in before, crack them open with fundamental discoveries and then move on to the next challenge while others picked up the pieces.

These distinct scientific styles were expressions of their distinct personalities. Günter would sit comfortably in his office reading while listening to Mozart, below a huge Roccoco-framed painting of the child composer sitting on the lap of the Austro-Hungarian Empress Maria-Theresa (to whom Günter was distantly related by lesser nobility). Axel in his office was lean, hyper-kinetic and constantly on the phone, keeping one step ahead of the competition via hot tips from his network of colleagues, dismissing most ideas as uninteresting and irrelevant while chain-smoking cigarettes with his feet up on his desk. He was brash, bold and irreverent where Günter was polite, polished and patrician. While Günter spoke a German-accented but precise English laced with colorful metaphors, Richard spoke like a street kid from Brooklyn in blunt language fragmented by frequent F-bombs. Günter's style was perfectly suited to the genteel, stately atmosphere of New York's wealthy Upper East Side (where Rockefeller was located), while Richard's was well-adapted to Columbia P&S's location in the crime- and crack-ridden

Washington Heights neighborhood at the northern tip of Manhattan (a place considered so dangerous in the mid- '80's that even Yellow Cab drivers from midtown refused to take passengers there).

At the time I joined the lab, Richard had just plunged into molecular neuroscience through a collaboration with Eric Kandel (and his biochemist side-kick Jimmy Schwartz) to understand the control of egg-laying in *Aplysia californica*. A team led by postdoc Richard Scheller had just cloned the gene encoding the precursor to Egg Laying Hormone (ELH) and made a big splash with a paper in *Science*[10] claiming that they had identified the molecular basis of an innate behavior, by suggesting that each of the multiple peptides predicted from the pre-pro ELH sequence might control a different step in the egg-laying sequence. I was amazed at how they were able to contextualize what struck me as a somewhat unsurprising finding (others like Ed Herbert had cloned and sequenced neuropeptide genes in mammals and discovered that their precursor proteins encoded multiple neuropeptides, like ELH) and make it sound like they had solved the genetic basis of behavior. But what did I know? I was just a *pisher* (Yiddish slang for a clueless but ambitious beginner) postdoc.

At the same time, Richard was using his recent ground-breaking discovery (made with Mike Wigler in his lab) of "transformation" – a technique to introduce foreign DNA into a cell line and use it to express and identify otherwise elusive genes based on their function[11] – to clone and sequence various types of cell surface receptors. These included the T cell surface protein T4[12] (cloned by Dan Littman and now called CD4), which later turned out to be the co-receptor for HIV. Using this technique, Moses Chao (a former Axel lab postdoc) cloned the gene encoding the low-affinity NGF receptor[13]. Axel would eventually unify his nascent interest in neuroscience with his nose (pun intended) for interesting cell-surface receptors to discover the genes encoding mammalian odorant receptors[14], for which he ultimately received the Nobel Prize with Linda Buck, the postdoc in his lab who did the bench work and who was my contemporary in the lab. (Ironically, the odorant receptors were not discovered using DNA transformation and expression-cloning, but rather through an unrelated approach involving PCR; see below.)

At first, I felt completely lost in the chaos of the overcrowded Axel lab, confined to a newspaper-sized patch of bench space shared with two other postdocs after having had a 9-ft bench all to myself in Günter's lab. My original idea to study AChR subunit assembly in mammalian cells transfected with the cloned receptor subunit genes crashed and burned due to conflicts with another postdoc in the lab. Having no clear idea of what else I could do, I pursued a project of Axel's design to understand the molecular diversity of cell types in the *Aplysia* abdominal ganglion using differential screening of cDNA libraries with radiolabeled cDNA probes made from single, dissected ganglion cell types[15]. This project was aimed at the right question, but ultimately failed due to the relative insensitivity of the technique. Indeed, the basic concept was the same as that underlying single-cell RNA sequencing, which has revolutionized the identification and classification of different neural cell types 30 years later. As usual, Axel was far ahead of his time.

Discouraged and tired of being mired in the inky viscous secretions of vivisected sea-hares, I decided to quit the project after 9 months and tried to pivot to a new direction that would take advantage of the cutting-edge molecular cloning techniques I had learned, so that I was not

starting all over again from scratch. I felt like a fighter pilot going down in flames, trying to pull myself out of a nose-dive before crashing. There were two major directions I considered. One was to use my training in cell biology to identify the components of pre-synaptic active zones and thereby understand the control of neurotransmitter release. In retrospect this was a good idea (it eventually won a Lasker Award for Richard Scheller, Thomas Sudhof and James Rothman and a Nobel Prize for the latter two). While it seemed an obvious project for someone with my training, I decided not to pursue it, for two reasons: First, there was no in vitro functional assay for neurotransmitter release, and I had been taught by Günter to believe that without one, such a cell biological problem could never be solved. (Ironically, Scheller, Südhof and Rothman discovered t-SNAREs, v-SNAREs and other proteins controlling synaptic vesicle release[16] without the benefit of any such *in vitro* functional assay). Second, I had also been taught that a process as fundamental as vesicle fusion should be the same in every cell type, and therefore instead of struggling to study it in complicated neurons I should use a more experimentally tractable system such as yeast, as illustrated by Randy Schekman's Nobel-winning work[17].

The other direction I considered, and ultimately chose, was embryonic neural development. Philip Grant, our neighbor in Woods Hole, was an embryologist who had influenced me to become a biologist and who encouraged me to investigate the molecular biology of development. Searching for a tractable experimental system for studying the development of neurons I discovered the neural crest, which gives rise to the entire peripheral nervous system as shown in elegant cross-species embryonic transplantation experiments by Nicole LeDouarin. This system seemed experimentally more tractable than the CNS, which was dauntingly complex, and potentially ripe for investigation using molecular techniques. I thought I could use the differential cDNA library screening techniques I had learned in my *Aplysia* project to identify marker genes that could label different types of neural crest derivatives and trace their lineage.

Chromaffin cells, chromatin structure and transdifferentiation

Through my attendance at the 1979 MBL Neurobiology Course in Woods Hole I had learned about the work of instructors Ed Furshpan, David Potter, Story Landis and Paul Patterson, then at Harvard, which had revealed evidence of remarkable phenotypic plasticity in sympathetic neurons: they could switch their neurotransmitter phenotype from noradrenergic to cholinergic in cultures treated with heart cell-conditioned medium[18] (which was later purified by Paul Patterson at Caltech and shown to be identical to the Leukemia Inhibitory Factor, LIF[19]). A similar and even more dramatic example of this phenotypic plasticity was work by Patterson's graduate student Allison Doupe demonstrating that fully differentiated adult adrenal chromaffin cells (endocrine cells) could be converted into sympathetic neurons in culture simply by treatment with Nerve Growth Factor (NGF)[20]. Strikingly, this dramatic phenotypic transformation could occur in individual cells in the absence of any cell division.

This example of transdifferentiation without mitosis caught my attention because a prevailing view at the time was that differentiation was inextricably coupled to cell division. One explanation for this tight coupling was proposed by Harold Weintraub of the Fred Hutchinson Cancer Institute in Seattle, a brilliant friend of Richard's whom I had met during his visits to Columbia. Weintraub had recently shown, using the globin (the protein component of hemoglobin) gene as a model, that latent changes in chromatin structure developmentally

preceded overt transcription, converting "closed," transcriptionally repressed chromatin into an "open" derepressed state that was transcriptionally quiescent, but accessible to cell type-specific transcription factors following differentiation[21]. Weintraub had suggested that such changes in chromatin structure could underly the phenomenon of embryonic "determination," in which apparently undifferentiated cells would express their normal differentiation program even after transplantation into an ectopic embryonic environment. Crucially, these changes in chromatin structure (now known as "epigenetic" changes) were thought to require cell division to permit changes in DNA methylation and histone remodeling that were dependent on DNA replication (although early experiments by Helen Blau challenged that idea). I found Weintraub's model, published in the same year as Doupe and Patterson's paper (1985), fascinating. The fact that adrenal chromaffin cells could differentiate into sympathetic neurons suggested to me that these fully differentiated endocrine cells might, due to their developmental history, maintain neuron-specific genes in an "open" chromatin configuration, transcriptionally quiescent but poised for expression in response to NGF. If so, it could provide an explanation for their transdifferentiation potential.

To test this hypothesis, I decided to use differential screening of cDNA libraries I constructed from sympathetic ganglion mRNA to identify genes expressed in sympathetic neurons but not in chromaffin cells, in the hopes that they would serve as early markers of transdifferentiation. I identified about a dozen such differentially expressed genes and showed that one of them, which I called "SCG10" (Superior Cervical Ganglion clone 10, later re-named--like all of the genes I originally discovered-- as "stathmin"), was a neuron-specific marker whose expression was low or undetectable in chromaffin cells, but which became induced during their in vitro transdifferentiation into sympathetic neurons in response to NGF. I learned how to dissect and culture primary chromaffin cells and transform them into sympathetic neurons from Allison Doupe, when I visited her at Harvard while she was a graduate student in Paul Patterson's lab. (Later, when Allison moved to Caltech to postdoc with Mark Konishi (see Volume 6), she and I became close friends and regularly kvetched over dinner at a cheap Chinese restaurant. It was a great personal loss to me when she passed away prematurely from breast cancer in 2014.)

Richard and I published our first paper together in *Cell* in 1985 describing these results, entitled "*Molecular Probes for the Development and Plasticity of Neural Crest Derivatives*."[22] Like my PhD work, the paper was less important for the results it contained than for the molecular techniques it introduced to the study of neural crest development, and the ideas about open chromatin and transdifferentiation raised in the Discussion. I have a vivid memory of Richard walking into the lab the morning after I had given him a draft of the paper proposing the "open chromatin" model for chromaffin cell plasticity, with my paper in his hand. He looked at me in a way that he hadn't before and mentioned that he found the idea interesting. I thought maybe he was considering the remote possibility that I was more than just a pair of hands. Later at Caltech, using DNase1 hypersensitivity to probe chromatin structure à la Weintraub, I confirmed that SCG10 was indeed maintained in an open chromatin configuration in chromaffin but not liver cells[23], consistent with our hypothesis. In the modern era, this concept has been confirmed and better understood in many cellular systems using more sophisticated markers such as antibodies to detect specific histone modifications associated with open vs. repressed chromatin. As the field has advanced our paper has been forgotten, but

I was glad that the idea (albeit one we proposed without any direct evidence) seemed to be on the right track.

From molecular to cellular approaches to neural development

During another visit to Woods Hole in the summer of 1984 I had an encounter that changed the trajectory of my postdoctoral research and influenced my scientific direction for years to come. At a softball game at Milfield Park interrupted by rain I met the craggy faced Martin Raff (see Volume 5), a brilliant cell biologist at University College London who had introduced, in a seminal 1983 Nature paper[24], the idea of using antibodies to cell-surface antigens to label different glial cell types and study their development in culture. I excitedly told Martin about my molecular data and ideas about open chromatin and transdifferentiation potential, and he told me that I was wasting my time trying to study this plasticity at the molecular level because I wouldn't learn anything that wasn't already known from studies of cell type-specific gene transcription in more tractable models, such as the globin gene. I was of course disappointed in his dismissal (although I later proved him wrong with the discovery of the Neural-Restrictive Silencer Factor (NRSF, aka REST; see below), which would never have been discovered from studies of the transcription of globin or other "standard" genes. At the same time, I could see the value of his studies of progenitor cells and their interactions with environmental signals, to complement the molecular work I had initiated.

I returned to Columbia determined to put this approach into practice. Doupe, Landis and Patterson had speculated in their 1985 paper reporting chromaffin cell transdifferentiation that this plasticity might reflect a common developmental progenitor to chromaffin cells and sympathetic neurons. I decided to test that hypothesis by isolating such progenitors from embryos and testing their developmental potential in vitro, using the type of approach Raff had pioneered. With help from Jane Dodd and Tom Jessell, who had recently moved to Columbia from Harvard and had a lab next door, I identified cell surface markers for these progenitors and was able to isolate them by FACS (Fluorescence Activated Cell Sorting), grow them in culture and show that single progenitor cells could divide and produce either sympathetic neurons or chromaffin cells, with NGF promoting the former and glucocorticoids the latter. These results were published in a second paper[25] with Axel in 1986, the year in which I moved to Caltech.

CALTECH: 1986-2026

My lab has meandered through many subfields of neuroscience during my 40 years at Caltech, reflecting my difficulty in choosing between interesting projects and directions. In the interests of brevity, I will highlight a few through-lines of research and their history, prioritizing those that have interesting twists and turns or associated anecdotes.

1986-2011. Neural development: stem cells, transcription factors and the neural crest

My initial research program at Caltech was shaped by the influence of two major scientific heroes (in addition to Richard Axel): Martin Raff and Harold Weintraub. The former guided me through stem and progenitor cell biology, the latter through chromatin structure and the transcriptional control of cell fate. Although these two fields were largely siloed from one another (I'm not even sure whether Raff and Weintraub ever met), my aim was to try to bring

them together and attack the problem of neural development at both the cellular and molecular levels. This multi-level approach has carried through my entire time at Caltech.

Neuropoiesis and neural crest stem cells

Paul Patterson, whose lab was adjacent to mine during this period, had made me aware of analogies between the developing immune and nervous systems through his pioneering work on cytokines. I expanded on this in a review article published in *Neuron* in 1989 entitled "*The Neural Crest Cell Lineage Problem: Neuropoiesis?*"[26], in which I suggested that the many different neural crest-derived cell types might develop from self-renewing stem and lineage-restricted progenitor cells in a manner analogous to hematopoiesis, as described by Irv Weissman at Stanford (another major influence) and others, suggesting the term "Neuropoiesis" to describe the model. I further suggested that this model could be tested in a manner analogous to hematopoiesis, by identifying specific surface markers for these stem and progenitor cells, isolating and purifying them by FACS and testing their developmental properties in clonogenic assays. Since we were at the time unable to track the progeny of single stem cells transplanted to a host embryo *in vivo* (as was done to map hematopoietic lineages), we relied instead on *in vitro* (as in petri dishes, not test tubes) clonogenic assay systems.

In those days, people sent reprint request cards by mail to authors. That review article must have struck a chord, because to my surprise it received literally hundreds of reprint requests (maybe over 500). Even Stephanie Canada, a Biology Division administrative assistant who sorted mail in the Division office, remarked to me "Wow, that must have been some article you wrote!" from the volume of reprint requests she received for it. Instead of feeling rewarded, however, I was disappointed that I was only able to muster that level of interest for a review/perspective article I had written, rather than for an actual primary research paper from my lab. It only reinforced my view of one of my limitations as a scientist, which was that my experiments were never as interesting (or important) as my ideas. Perhaps that was because my father was a theoretical physicist who trained with a former student of Einstein's – who famously said "Imagination is more important than knowledge." Ultimately, of course, I have no one to blame but myself.

At least I tried to put my money where my mouth was. Our major objective in this period was to see if we could isolate an undifferentiated progenitor cell from the neural crest, and test whether it possessed the two cardinal properties of stem cells: multipotency and self-renewal. This project was carried out by Derek Stemple, one of my first graduate students who was both brave and creative. After 5 years of hard work, Derek and I published our paper on the isolation of a self-renewing multipotent stem cell from the neural crest in 1992 in *Cell*—the first *Cell* paper from my laboratory[27]. This was the first demonstration that such stem cells existed in, and could be isolated from, an embryonic vertebrate nervous system. More important than the particulars it reported, it helped fire up the field of neural stem cell biology which exploded over the next 5 years. Of course, once a stem cell was finally isolated from the central nervous system, Derek's landmark paper was rapidly forgotten (since, as I liked to say, "nobody gives a s--t about the peripheral nervous system"), but I'm glad we helped to jump-start the field.

The long gestation of the work and the obstacles encountered along the way show how mercurial and unpredictable this type of research can be. Derek had isolated neural crest cells

using a monoclonal antibody to the low-affinity NGF receptor and had cultured them on a fibronectin substrate. The cells readily differentiated into what looked like Schwann cells but try has he might, Derek was unable to coax them to differentiate into neurons. We were ready to throw in the towel and write it up as the isolation of a committed glial progenitor, when one day I came back from a meeting where someone reported successfully culturing chick peripheral neurons on a polyornithine-laminin substrate. It occurred to me that maybe neurons were being born but couldn't attach or survive on a fibronectin substrate, so I suggested to Derek that he try adding poly-L-lysine and laminin to the substrate. Miraculously, when he did so neurons miraculously appeared in addition to glia, and we were off and running.

Why it worked I still don't know to this day, but the episode reinforced the hit-or-miss, alchemical nature of this kind of work. In fact almost a decade later (2001), when the public finally started to pay attention to stem cell research thanks to the first reports of human embryonic stem cells, I published an Op-Ed in the NY Times entitled "*The Alchemy of Stem Cell Research*," to emphasize how much the discovery of the signals necessary to coax stem cells to differentiate into a desired cell type depended on educated guesses and trial-and-error. Fortunately, in our case the educated guesses turned out to be good ones, and eventually Nirao Shah (the second graduate student in my lab to work on neural crest stem cells) published two beautiful papers in *Cell* identifying growth factors that pushed the stem cells to differentiate into autonomic neurons (BMP2/4), fibroblasts (TGF) or Schwann cells (Glial Growth Factor, or GGF/Neuregulin-1 as it was also called)[28, 29]. Importantly Nirao showed – for the first time in any stem cell system—that these signals acted on the stem cells by instruction (i.e., inducing them to choose one differentiated fate at the expense of others) rather than by selection (promoting the selective survival of one cell type in a mixture of different cell types generated at random by the stem cells – which was the prevailing view of hematopoiesis at the time). Martin Raff, at least, thought this was interesting and important.

Neural cell fate determination genes: the cloning of *Ascl1* (aka MASH1) and the *Neurogenins*

One of the key next questions was to understand how and when the progeny of a neural stem cell started to become different from one another. Nirao's results suggested that this process probably began hours or days before overt neuronal differentiation was observed. Apparently, some stem cell progeny possessed a latent neurogenic potential that was absent in other, morphologically indistinguishable cells. We wanted to find a marker that would make such an invisible state visible, so we could study the lineage restriction process. In addition, we hoped that such a marker might play a causal role in lineage restriction.

In the late 1980s, Hal Weintraub published a landmark paper identifying a member of the recently identified basic helix-loop-helix (bHLH) family of transcription factors, which he called MyoD, which first became expressed in committed muscle precursors before they showed any other signs of overt differentiation, and whose forced expression was able to convert almost any cell type into a myoblast[30]. This discovery, which sent shock waves around the world, was the first identification of a transcription factor that caused *determination* (invisible commitment to a particular fate) rather than overt *differentiation*. Had Hal not died tragically of glioblastoma in 1995 at the age of 49 he almost certainly would have shared the 2012 Nobel Prize with Shinya Yamanaka and John Gurdon, for their demonstration of the pleuripotency of embryonic stem

cells and the transcription factors that conferred this property. Hal's discovery of MyoD was electrifying; I was certain that there must be analogous determination factors for neurons and glia, and that if we could find them, they would help us unlock the secrets of neural crest stem cell fate determination. The question was, how to get our hands on them?

My first (derivative) guess was that such neurogenic determination genes were likely to be related to MyoD; i.e., to be members of the bHLH family. Work by David Baltimore at MIT reinforced this idea by identifying other bHLH factors (called E-proteins) that controlled the differentiation of specific immune cells. Using MyoD or E-protein cDNA probes to screen neural cDNA libraries at low stringency seemed messy and likely to get us nothing but lots of false positives. At this point, both technical advances and serendipitous events occurred that gave us a new way to try to discover these molecules. The first was the development of the polymerase chain reaction (PCR) by Kary Mullis, a technique that allowed one to amplify vanishingly small amounts of DNA by orders of magnitude for further analysis (for which Mullis won the 1993 Nobel Prize in Chemistry). The second was the use of PCR to clone new members of a given gene family by amplifying cDNAs with "degenerate" DNA primers, short pieces of "starter" DNA that could in theory hybridize to sequences shared by different members of a gene family with sufficient stability to permit PCR amplification. (This was the technique that eventually allowed Linda Buck and Richard Axel to clone the olfactory receptor gene family.) This seemed like an ideal way to try to clone MyoD relatives that served an analogous function in neural precursors.

At the time, however, very few labs were set up to use this new technique. Fortunately, one of those was that of Melvin Simon, who had recently moved his lab to Caltech from UC San Diego. A postdoc in his lab named Tom Wilke had set up this technique. Coincidentally (or perhaps prophetically) Tom's wife Jane Johnson, who had worked as a visiting graduate student in Barbara Wold's lab at Caltech on muscle differentiation, decided (with Barbara's encouragement, another debt I owe her) to join my lab as a postdoc. In a match clearly made in Heaven, this put us in a unique position to screen for neural friends and family of MyoD.

Using cDNA isolated from an immortalized sympathoadrenal progenitor cell line (called "MAH" cells) made by Susan Birren, another postdoc in my lab, Jane threw dozens of MyoD-related degenerate primers (and thousands of dollars) into PCR reactions to try to amplify a "NeuroD," without avail. We were about to give up in our hunt for the white whale when Kai Zinn (a tall, lanky Assistant Professor from Los Alamos NM who had recently joined the Caltech faculty) came back from a visit to his postdoctoral mentor Tom Maniatis at Harvard with some interesting news. Tom was interested in studying muscle development in *Drosophila* and had isolated a fly homolog of MyoD. Strikingly, Kai told me, the bHLH sequence of fly MyoD was more homologous to that of mouse MyoD than it was to other fly bHLH family members. The latter included *achaete* and *scute*, two obscure and unpronounceable genes that controlled the development of neural precursors in the fly PNS. Kai suggested that maybe instead of trying to use degenerate PCR to isolate neural homologs of MyoD, we should be trying to isolate neural homologs of *achaete* and *scute*. This was undoubtedly among the best advice I have ever received from any faculty colleague in my 40 years at Caltech, and Kai laconically tossed off the idea like a suggestion to try a new breakfast cereal. Electrified, I knew Kai had to be right.

I ran up the steps of my building back into the lab and excitedly told Jane that she should throw out all her MyoD and E-protein primers and immediately try degenerate primers for *achaete* and *scute*. She did so, and within a week she had cloned two *achaete-scute* homologs from MAH cell cDNA. Like MyoD, the bHLH sequences of these homologs, which we called MASH (for Mammalian Achaete-Scute Homolog)-1 and -2 (in honor of my then wife's favorite TV show), was more closely related to the fly *achaete-scute* bHLH sequences than it was to any other mammalian bHLH sequences including MyoD (explaining why we had failed to find them using MyoD PCR primers)[31]. This finding, together with Tom Maniatis's results for fly MyoD, indicated that the cell-type specific expression of bHLH factors and their amino acid sequences was conserved across 500 million years of evolution.

This result had ramifications far beyond simply identifying candidate neural detemination genes in vertebrates: it indicated the existence of a fundamental transcriptional code for cell type, analogous to the transcriptional code for body axis position shared by fly and mammalian homologs of the homeobox gene *bithorax* and its family members as discovered by Robb Krumlauf and others. With a mixture of excitement and terror, I realized that we had stumbled on an important and potentially universal principle underlying the development of different cell types and tissues. Something that not only developmental neurobiologists, but *everyone* in the field of developmental biology should care about. I even remember Christiane Nüsslein-Vollhard leaning forward in her chair and squinting at my slide when I showed the sequence alignment between the *MASH* and *Achaete-Scute* genes at a meeting in Heidelberg. Suddenly, my parochial work on the neural crest had acquired a broader relevance.

The discovery of the *MASH* genes (now renamed *Ascl1* and *2* by the human genome nomenclature committee in their infinite wisdom) excited the vertebrate neural development community, not only because of their potential importance for neural fate determination, but also because it made everyone realize that the whole network of *Drosophila* neural developmental control genes, including *Notch*, *Delta* and their ligands as well as other bHLH factors, could be used as hooks to fish out their mammalian homologs. All of a sudden, instead of flailing about trying to guess what genes might be important for mammalian neural development, one could take advantage of the powerful unbiased, systematic genetic approaches available in flies to provide candidates for vertebrate neural developmental control genes. Interestingly, at the time (1990) the prevailing view in the neuroscience community (as articulated in textbooks like Dale Purves's (see Volume 11) and Jeff Lichtman's (see Volume 14) classic *Principles of Neural Development*) was that vertebrate and invertebrate brains developed according to fundamentally different principles: invertebrates used a cell-intrinsic lineage-based program, known tongue-in-cheek as the "European plan" (you do what your ancestors did); while vertebrates used cell-extrinsic signals to direct the differentiation of otherwise plastic cells, known as the "American plan" (you do what your neighbors do). Our results showed that simply wasn't true – at least at the molecular level.

Ironically and frustratingly, the one obvious experiment we tried immediately after cloning the *MASH* genes – to mis-express them in fibroblasts and see if they would convert those cells into neurons—didn't work. It would take years of trial-and-error experiments by other labs including Marius Wernig's, as well as the isolation of additional vertebrate neural bHLH genes such as the *neurogenins* (discovered by my lab) and the *atonal/NeuroD* family (cloned by Huda

Zoghbi, see Volume 14"), to find a cocktail of transcription factors that could be used to re-program human fibroblasts to neurons à la MyoD[32]. While I sometimes wish my lab had stuck with the molecular alchemy long enough to get it to work, the one satisfaction I have is that *Ascl1* and *neurogenin* have turned out to be useful neural re-programming factors[33]. Hopefully one day this technology will be applied to treat human neurological diseases.

Silencing is golden: how neuron-specific gene expression is achieved.

In the 1980s the prevailing view among molecular biologists was that cell type-specific gene expression was achieved by specific "enhancer" elements: short DNA sequences located in the region upstream (5') of the gene's coding sequence that were recognized and bound by cell-type specific transcription factors, which recruited RNA polymerase II to promote transcription. (These cell type-specific transcription factors were in turn thought to be activated by a combinatorial code of factors during the cell's developmental history and then, once activated, to maintain their own expression through positive autoregulation.) The advantage of this mechanism is that the same enhancer element could be used over and over in the terminal differentiation genes specific to that cell type's function. In a muscle cell, for example, this "gene battery" (a term coined by my late Caltech colleague Eric H. Davison) would encode proteins such as actin, myosin, troponin, tropomyosin, etc. Thus, as soon as the "master regulator" transcription factor for muscle was turned on, all the genes in the battery would turn on as well. In this respect MyoD and its relatives, some of which were discovered by Barbara Wold, appeared to fit the bill as "master regulators" of muscle; the enhancer element it bound, called the "E-box," was present in and controlled the expression of many muscle-specific genes.

Given Richard Axel's interest in the mechanisms of transcription, my training in his lab aroused my interest in the problem of how neuron-specific gene expression was controlled; I saw it as another potential bridge to build between molecular biology and neuroscience. But at the time most molecular biologists (including Axel) simply assumed that a similar mechanism would operate in neurons; why should they be different from any other cell? While the discovery of a neuron-specific enhancer and its cognate master regulator might provide some experimental utility (e.g., in allowing neuron-specific gene expression from viral vectors), their identification would be unlikely to yield any fundamentally new insights into the control of transcription that had not already been established from studies of model cell type-specific genes such as globin or pancreatic elastase. Therefore, the thinking went, studying the control of neuron-specific gene expression would be a waste of time, and Richard discouraged it.

I was sensitized to this neuro e-centric view from my training in Blobel's laboratory. Although my PhD thesis on the synthesis and membrane integration of the AChR subunits might excite some neurobiologists, in Günter's view they revealed no new fundamental concepts in cell biology. It was a perspective I was to encounter many times in my career: the cellular and molecular mechanisms underlying the development and function of neurons were assumed to be the same as in every other differentiated cell type, and while perhaps of parochial interest to neurobiologists (who were viewed as an insular and myopic bunch), their study was unlikely to reveal any new principles of cell function (Ironically, even when specialized neuronal proteins were shown to control processes like synaptic vesicle release[16], since those mechanisms did not generalize to other cell types they were not considered "fundamental." I guess you can't win.)

Despite this discouragement and dismissal, I thought that understanding the mechanism of neuron-specific gene expression would still be worth the effort, principally for two reasons. First, neurons presented a special case of transcriptional control because of the extreme diversity of neuronal subtypes. Each neuron type expressed a combination of genes that included both generic pan-neuronal (e.g., the sodium channel), and cell type-specific (e.g., neuropeptide) genes. On the one hand this might be achieved by having two parallel transcriptional control systems, one for pan-neuronal and the other for cell type-specific genes. In that case the research might indeed identify a master regulator of the neuronal phenotype. On the other hand, even "pan-neuronal" genes might be under the control of many different cell type-specific transcription factors in different neuronal cell types, in which case the search for a master regulator would be a fool's errand. There was only one way to find out. And who knew? -- there might after all be special transcriptional mechanisms for neurons that had not yet been identified by the study of tissue-specific transcription in other non-neuronal systems.

The second reason for chasing this potentially wild goose was to better understand neural development. Eric Davidson (who was quite powerful and influential at Caltech during that time) had argued that the "right" way to approach this complex problem, at least in a system lacking traditional mutational genetics such as the sea urchin embryo (on which he worked), was to begin by identifying the transcriptional regulators that controlled expression of lineage-specific "end-state" genes, and then to work "backwards" in developmental time to identify the regulators of the regulators, and their regulators in turn, and so on. In principle one could then use the developmentally earliest regulators both as markers, to identify otherwise undifferentiated embryonic cells that had already "chosen" a neuronal fate, and also as a handle to understand the molecular mechanism of this developmental decision.

So, despite warnings from people like Axel and Raff, I pursued this potentially spurious holy grail using *SCG10*, the neuron-specific marker gene I had identified in my sympathetic neuron vs. chromaffin cell screen in Axel's lab. *SCG10* was abundantly expressed from the earliest stages of neuronal differentiation in what, as far as I could tell, was every neuron type. Later, *SCG10* turned out to be a member of the "stathmin" gene family, which encodes microtubule-severing enzymes as shown by Tim Mitchison. SCG10 is a membrane-tethered isoform of stathmin, which we found accumulated in the growth cones of nascent embryonic axons, perhaps to prevent the extension of axonal microtubules into this region so they could be replaced by the terminal actin-filament network. It was never our intention to understand the function of SCG10; for us it was just a marker. (As Peter Walter once said disparagingly of molecular biologists who used what Günter called "their gun-shot approach"--a mangled reference to "shotgun" DNA sequencing--"The Lord forgive them, for they know not what they clone."

Through almost a decade of work that began virtually as soon as I arrived at Caltech, we discovered a mechanism for the transcriptional control of *SCG10* expression that had not yet been described by the "professional" molecular biologists in any of their favorite model genes. We found no evidence that the SCG10 gene contained a positive-acting neuron specific enhancer element recognized by a neuron-specific "master regulator" transcription factor -- which was the boring result that my molecular biology colleagues had predicted. Instead, we found evidence of a *de-repression* mechanism, in which the expression of neuron-specific genes

was turned *off* in non-neuronal tissue by a DNA transcriptional repressor element (or silencer, as we called it) that was bound by a protein that, at least in developing embryos, was present in every tissue *except* neurons. Even in the nervous system, moreover, this silencer was present in neuronal precursors in the ventricular zone of the spinal cord and was turned *off* as the cells migrated and differentiated into the marginal zone to become motor and inter-neurons.

We discovered this mechanism by first identifying the *cis*-acting DNA repressor element in the *SCG10* upstream region, which we named the NRSE (the Neural-Restrictive Silencer Element)[34]. Subsequently, using a radioactively tagged NRSE as a label, my graduate student Chris Schoenherr identified and cloned the silencer protein that bound to it, which we called NRSF (the Neural-Restrictive Silencer Factor)[35]. A similar DNA repressor element was identified by Gail Mandel (see Volume 12) in the type II sodium channel gene and named "Repressor Element I" (RE-1). Using labeled RE-1 as a tag and a similar strategy as we did, she independently cloned NRSF, which she named the "Repressor Element I Silencing Transcription factor," or REST[36].

Remarkably, our initial genome searches[37] (and more comprehensive later work by my Caltech colleague Barbara Wold[38]) revealed that most genes containing the NRSE/RE-1 in their non-transcribed sequences encoded neuron-specific proteins, many of them isoforms of more broadly expressed proteins. We hypothesized that since many of these neuron-specific genes had apparently evolved by duplication and modification of ubiquitiously expressed genes (e.g., neuron-specific tubulin, neurofilament, neuron-specific enolase, etc.), a parsimonious explanation was that it might have been more economical to evolve a silencing mechanism to keep these genes turned off in non-neuronal tissues, than to have to evolve *both* a new, positive-acting neuron-specific transcriptional enhancer *and* mutations to inactivate the ubiquitous enhancer(s) controlling non-neuronal expression (which presumably were copied when these broadly expressed genes were initially duplicated).

We were the first to report the isolation and sequence (albeit a few amino acids short of the C-terminus) of the gene encoding this silencer protein in *Science*, ahead of Mandel's publication by several months. As the initial discoverer, we had earned the traditional right to baptize the gene and its encoded protein as NRSF. However, several months later someone from the Human Genome Gene Nomenclature Committee called me up and said that the name "*Nrsf"* had already been assigned to another unrelated gene, and would I please suggest a new, different name for it. When I struggled to come up with an alternative moniker on the spot, the Gene Nomenclature representative asked whether I would just approve using REST, Gail Mandel's name for it. Being young and naïve, to my everlasting regret and chagrin I immediately said 'yes,' not realizing that in so doing I had not only consigned the name "NRSF" to the trash-heap of forgotten gene names, but also inadvertently assigned Mandel spurious credit for the initial discovery of the silencer protein. As Nicholas Wade once wrote in the *New York Times* when describing the conflict between Luc Montagnier and Bob Gallo over naming the virus that caused AIDS, "In science, *as in primitive societies* (italics mine)*,* to name an object is to own it." That accidental transfer of ownership, as well as our finding that a gene knockout of *NRSR/REST* produced no obvious defect in neural development caused me, at first reluctantly, to abandon further studies of the protein several years later and leave the field in the capable hands of Gail Mandel. That decision (as well as other factors as discussed below) presaged my eventual

abandonment of transcription factors, stem cells and neural development and my shift into neural circuits and behavior.

The one lasting satisfaction I derived from this otherwise Pyrrhic victory is that an excellent, highly specific monoclonal antibody to NRSF that we developed eventually proved useful to my colleague Barbara Wold (a fellow Axel lab alum and my counselor and confidante in all things transcriptional at Caltech) in her development of the widely used ChIPseq technology. Her original paper describing this invention[38] has been cited almost 4,000 times – many more than any paper I have ever written. Fortunately, Barbara graciously referred to the protein throughout her ChIP paper as NRSF (pressuring the editor of *Science* to allow her to do so), so that our role in the discovery of this now-famous protein is not foreover lost to history.

1998-2026: neural circuits, innate behavior and emotions

Changing fields

I think it's safe to say that I have done my best and most original work when opening up or getting into a new field. It's exciting, stimulates the imagination and creates a virtually unlimited horizon of opportunities, a completely empty sandbox with no one to play in it except yourself. I dislike working in a crowded and competitive field, always worrying that some unknown competitor is breathing down my neck and turning every project into a race. So it happened in 1998 when, 6 years after we had identified the neural crest stem cell, Jamie Thomson of the University of Wisconsin-Madison dropped the bombshell report of the first isolation of a human embryonic stem cell line, initiating a gold-rush into the field of stem cell biology. Suddenly everyone was studying stem cells – embryonic stem cells in particular, because of their potential therapeutic applications to human disease. Predictably, this rush was accompanied by some questionable science, and there was a flurry of eventually discredited papers in high-profile journals reporting the conversion of stem cells from blood into brain, brain into bone, etc. It was proving difficult to keep up in this race while doing science in the rigorous but painstaking way required to maintain the high scientific standards I had internalized from Richard Axel. More importantly, it had become clear that no one in the stem cell field gave a s--t about neural crest stem cells, while no one in the *neural* stem cell field gave a s--t about the stems cells for peripheral neural nervous system. I decided it was time to move on to a new field – just around the time that California voters approved a proposition committing $3 billion to stem cell research over a 10-year period. I remember waking up one night at 3 AM from a fitful sleep, looking bleary eyed into the mirror and asking myself "are you out of your f---ing mind?!" Yes.

"Genes don't control behavior; neural circuits control behavior" – R. Axel

It was around this time (the mid-'90s) that Richard Axel had begun to use molecular techniques to trace the connectivity of neurons expressing the odorant receptor genes that he and Linda Buck had isolated in 1991. Up until this time, most molecular biologists working in neuroscience, such as MIT's Susumu Tonegawa, were taking the approach of knocking out specific genes in mice and examining the behavioral phenotype. This "reverse genetics" approach was one way to address the fascinating question of how genes control behavior (which had drawn pioneer molecular biologist Seymour Benzer into the field using *Drosophila*). It was made possible by the development of technology for targeted gene knockouts in mice

using homologous recombination in embryonic stem cells, by Mario Capecchi (who won the 2007 Nobel Prize for this contribution). The pages of *Cell* began to fill up with such papers.

By the mid '90s, however, it was becoming clear that even with "conditional" (brain- or region-specific) knockouts, it was going to be difficult to understand how such engineered mutations produced their behavioral phenotypes in adult mice, because the knockout could disturb the brain's early development or function (or both) and could impact the latter in many indirect ways. Without knowing the circuits in which the gene was acting, and how those circuits developed and controlled the affected behavior, interpreting such experiments was largely guesswork. Even the demonstration that a hippocampus-specific knockout of the *calcium/calmodulin-dependent protein kinase (CamK) II* gene affected both LTP in acute brain slices and spatial learning in freely moving animals didn't formally prove (contrary to some claims) that hippocampal LTP is necessary for spatial learning; the two mutant phenotypes could in principle have been independent and causally unrelated.

What, then, could molecular biologists like me contribute to the understanding of behavior? My epiphany came when Richard Axel introduced a lecture I attended with the bold assertion that "Genes don't control behavior; neural circuits control behavior." He then proceeded to elegantly show how the tools of molecular biology could be applied to map and manipulate neural *circuitry*, rather than to perturb a gene's *function*. Crucially, rather than using homologous recombination in ES cells to knock *OUT* a gene, he applied Capecchi's technology to knock *IN* to a particular gene locus an anatomical marker, such as bacterial ß-galactosidase fused to the microtubule-associated gene *tau* (aka "tau-lacZ"), to attach it to axonal microtubules. This placed the expression of tau-lacZ under the control of *all* the genomic transcriptional regulatory elements that governed the specific *expression* of that gene in particular neurons and allowed one to trace the projections of those neurons using a blue histochemical enzymatic reaction for lacZ expression. This elegant strategy bypassed the laborious traditional approach of first identifying in a piecemeal manner specific transcriptional enhancers for a given gene, which could take years and was frequently hit-or-miss.

This revolutionary approach was spectacularly applied to individual odorant receptor genes by Peter Mombaerts (then a postdoc in Axel's lab) to show that all of the olfactory sensory neurons (OSNs) in the nasal epithelium that expressed a given odorant receptor gene sent their axons to a common glomerulus in the olfactory bulb, and moreover that each OSN projected to a different glomerulus, thereby revealing a spatial map of odor specificity in the bulb[39]. Using the same knock-in strategy Axel later showed that electrically silencing such OSNs, by specifically expressing the inwardly rectifying potassium channel gene *Kir2.1* (a technique, like tau-lacZ labeling, borrowed from *Drosophila*), caused a disruption of these projections[40]. This implied that spontaneous electrical activity in these neurons was required to form this olfactory map. I found these experiments very exciting, not only for the insights they yielded into olfaction but also for what they portended for the field more generally.

When, in the late '90s, I began to discuss with Richard (whose advice and mentoring I frequently sought throughout my career) my plans to switch from neural development into neural circuits and behavior, these powerful genetically based techniques for circuit mapping and manipulation were in their infancy (at least in mice; *Drosophila* was far ahead at the time).

It seemed like one contribution I could make would be to build on this approach to develop variations of these techniques, such as using the activity-dependent regulatory elements in the c-*fos* gene to drive expression of tau-lacZ, so that one could specifically map the projections of only those neurons that were *active* during a particular behavior. (This idea was, like many of mine, too far ahead of available technology at the time and only became truly feasible beginning in 2013, after Liqun Luo developed the "TRAP" technology to stably mark active neurons using a *c-fos* knock-in of Cre recombinase[41]).

While Richard was generally supportive of this approach, he wisely suggested that I avoid pure technology development and instead develop and apply these methods as they were needed, to a biologically interesting and important problem. "Pick a behavior and map the circuit," he advised. Since Richard was usually right, I followed his advice. The question was *which* circuit, and *which* behavior?

A fitful foray into fear

The molecular labeling of specific neural circuits ought to be particularly useful, as Richard had shown, for mapping innate behaviors. Presumably these behaviors, such as mating, aggression and parenting, were controlled by developmentally and genetically specified neural circuits. Since such "hard-wired" circuits were likely to be anatomically invariant across individuals as well as molecularly specified, the use of molecular genetic techniques to map neural pathways seemed most likely to be useful in the case of such instinctive behaviors.

In thinking about relatively virgin territory where I could begin to apply such approaches productively, I considered that it would be wise to choose behaviors that were robust and which had been studied intensively using classical neuroanatomical techniques, but where important unanswered questions remained. It was the same strategy that had originally drawn me to focus my studies of neural development on the neural crest, rather than on the more complex and less accessible CNS. To avoid competing with Richard and his progeny (such as the formidable Catherine Dulac, who like me had moved into circuits and behavior from studying neural crest development with Nicole LeDouarin and who had already identified a family of candidate pheromone receptors in Axel's lab), I decided to stay away from instinctive reproductive behaviors. Instead, I chose to focus on fear (or more accurately, freezing behavior). While a great deal had been learned about the neural circuits underlying "conditional" (or "conditioned") fear, by neuropsychologists such as Joseph LeDoux and Michael Fanselow, much less was known about circuits for innate fear. If one could map such circuits, I reasoned, it would not only be intrinsically interesting but could also yield insights into the question of whether innate and learned fear were completely parallel circuits or shared a final common pathway. It could also be relevant to understanding how brains encode emotion, and thus to mental illness.

The first step was to find someone with the skills to pursue this project. So in 1998 I took a risk and hired Raymond Mongeau, a French postdoc in Michael Fanselow's lab at UCLA with experience in the anatomical, behavioral and pharmacologic techniques used to study conditioned fear. The next step was to identify a stimulus that could elicit robust innate fear (freezing) responses in laboratory mice. Little did I know how difficult this would prove to be.

Soon the lab was filled with the retch-inducing stench of coyote urine and synthetic predator odors such as TMT, which were used by farmers to keep agricultural pests away from their crops. Unfortunately, our standard laboratory C57BL6/J mice seemed completely indifferent to such smells. We thought we might have better luck if we used a live predator. But our campus vet wouldn't allow us to bring my cat into the lab to present to the mice. Instead, one day we brought a cage of mice upstairs to the lab of the great neuroethologist Mark Konishi, who was rearing owls to study sound localization. When we placed the cage in front of the young birds, they immediately went batshit (or more accurately owlshit) crazy -- screeching, flapping their wings and madly pecking and grabbing at the cage with their tiny talons. Meanwhile the mice couldn't have cared less and simply continued to go about their normal business of grooming, eating, urinating and defecating.

Apparently, decades of cushy laboratory living had literally bred the fear of natural predators out of these rodents. In desperation, we imported a strain of wild mice that had only recently been introduced to the laboratory from the Institute of Genetics in Mishima, Japan. These animals seemed an altogether different species from our docile lab mice, capable of jumping many feet into the air and of darting around at lightning speed. After having to change their cages in large garbage cans to prevent the mice from escaping, and spending hours trying to catch a lucky creature that managed to elude our grasp, we decided they were too much trouble to work with and abandoned them. It was back to the drawing board.

To make a long story short, my first foray into behavioral neuroscience produced a less-than-earth-shattering publication[42] describing the pattern of neuronal activity throughout the brains of mice during responses to aversive ultrasonic sweeps (18-22 kHz), a stimulus which we had found could evoke innate avoidance and risk-assessment behavior, if not outright freezing, in naïve mice. While it was the first effort to map brain-wide patterns of neural activity at cellular resolution during an innate behavior using *in situ* hybridization for *c-fos*, it fell far short of making the splash I was hoping for, being rejected even by *Neuron* and published in *J. Neurosci*.

Making this switch in fields was the hardest thing I've ever done, professionally. I had been a professor at Caltech for 15 years, and suddenly it felt like I was starting all over again as an assistant professor. Talented postdoc candidates interested in stem cell research stopped applying to my lab. I stopped getting invited to stem cell meetings and lost contact with many of my colleagues in the stem cell field. I got no invitations to any meetings on fear or the amygdala and nobody applied to my lab to work in this area. After having been one of the main players in the stem cell field, I had descended into obscurity. While I was trying to continue to sail forward, the wind was in my face rather than at my back. It was like going through a painful divorce, and I began to wonder if I had made a serious mistake.

Do flies have feelings?

Seymour Benzer, giant of genetics and founder of fruitfly neuroscience, stood up to greet me as I walked into his cramped office at Caltech. Walled in behind mountainous stacks of books and papers on his desk, he rose slowly and trundled his aging frame carefully around his precarious piles to greet me as I entered the room, then sat in a peeling gray metal lab chair to face me while motioning to me to sit on a similar stool facing him. A gigantic, magnified photograph of a fruit fly's head adorned the wall behind him, next to a poster from a remake of

the movie "The Fly" starring Jeff Goldblum. 'Be Afraid – Be Very Afraid,' said the tag line. Benzer, a transplant from New York to Southern California, retained his Brooklyn accent, pale complexion and an East-coast Yiddishkeit manner that managed to persist in Pasadena despite its white-bread all-American character. (San Marino, where Seymour lived, had been the home of the infamous John Birch Society during the early 20th century and faint echoes of its anti-semitic ethos could still be heard, even at Caltech.) Draped in a dirty labcoat, pockets bulging with forceps, notecards and hemostats, a tartan tie and dangling spectacles adorning his neck, he sat like a Jewish Buddha, a godfather of genes and behavior, waiting for me to speak.

After telling a Borscht-belt joke to break the ice, I waited until the right moment to deliver what I thought would be exciting news to him. It was Seymour who had personally recruited me to Caltech in the mid-1980's, Seymour who had convinced me to leave my home in Manhattan by driving me around LA in his beat-up 1962 red Dodge Dart convertible and showing me the gritty underside of the city, with its diverse hole-in-the-wall ethnic restaurants. Even though I worked on mice, Seymour kept telling me that one day I would "see the light," as he put it, and switch to fruit flies. Now, the difficulties I was experiencing as I struggled with neural circuit research in mice made me seriously consider whether I should start working on flies instead.

It was early days and rough going; we had problems generating BAC transgenic mice, and important new techniques like optogenetics hadn't been invented yet. Mice were slow and expensive: they had a 12-week generation time, with months required to breed the large numbers of animals needed for behavioral experiments. Flies were cheap, fast (a 12-day generation time) and plentiful. Moreover, the field already had developed genetic techniques for turning neurons on and off (such as the dominant temperature-sensitive *shibere* mutation used to inhibit neurotransmitter release), which were not yet available in the mouse. I thought it might be time to "see the light" and switch to flies. The only question was whether flies could be used to study primitive emotions.

"So Seymour," I asked, "do you think flies have fear?"

Seymour looked at me quizzically, like he couldn't believe I was serious. "Of course, they do," he said, as if nothing could be more obvious. Yet I'd asked the same question of other colleagues, such as the engineer and neuroethologist Michael Dickinson, and the unequivocal answer was along the lines of: 'I doubt it-they're just little, very sophisticated, robots.'

Seymour had once told me that if you ask your colleagues for advice about an experiment you're thinking of doing, and everybody says 'don't do it,' clearly you shouldn't. If they all say 'of course you should do it," that means it's too obvious and therefore you also shouldn't do it either – everybody else will too. But if half the people you ask say you *should* do it, and half say you *shouldn't* do it, then you should definitely take the risk and do the experiment.

"How do you know that flies have an internal state like fear, and not just robotic escape reflexes?" I pressed. Seymour gave me a "do you really want to hear this?" look, cleared his throat, and told me an anecdote about an unpublished observation he'd made in his lab.

In the '70's, Seymour had surprised the world by showing that fruit flies can learn and remember to avoid an odor that was previously associated with a footshock. To do this he had built a clever little apparatus called a "T-maze," to test flies' preference for one odor versus

another, after first pairing one of the odors with a foot-shock. While doing these experiments, Seymour noticed that after training one batch of flies in the "shock tube" (which had a thin copper grid on the floor to conduct electricity), the next batch of flies tended to avoid entering the shock tube – even before any shock was delivered. It seemed as if the first group of shocked flies had left behind some kind of odor or residue that was innately aversive to other, naïve flies.

This anecdotal result caught my attention. Famous experiments by the Nobel Laureate Karl von Frisch in the late 1930's had shown that when stickleback fish are stressed, they release an odor from their skin that other fish avoid. He called this odor "*Schreckstoff*," (literally, "fear substance,") and suggested that it might be a kind of alarm signal that fish emit when exposed to a predator, to communicate the presence of the threat to other fish. Seymour's observation in the T-maze suggested that maybe shocked flies emitted a kind of "Schreckstoff" as well. If so, then the powerful genetic tools available in *Drosophila* might allow us to identify this odor and the neurons that detect it, in a way that von Frisch and his colleagues had been unable to do.

I thanked Seymour for the tip, left his office, filed his anecdote away in my brain for future reference and went back to struggling with my not-so-fearful mice. Fast-forward to two years later, when a young student named Greg Suh approached me to ask if I would take him on as a postdoctoral fellow in my lab. Greg had received his PhD at UCLA with Larry Zipursky, himself a former postdoc of Benzer's, working on the development of neuronal connections in flies. Larry had recommended Greg highly. I saw this was a chance to start working on flies, and accepted Greg into my lab immediately.

For Greg, moving to a lab that had no previous experience with *Drosophila*, and setting the system up from scratch, was taking a major risk: he could easily have gone to an established fly lab instead. To de-risk the move a bit, I suggested that he could start by following up on Seymour's "Schreckstoff" observation, in collaboration with Benzer's laboratory. For Greg –as well as for me - the chance to collaborate with Seymour, the founding father of *Drosophila* neuroscience who (even at 80) still ran an active and productive lab, was a dream come true.

After Greg arrived at Caltech, I excitedly called Seymour on the telephone. I explained that Greg had come to my lab to start working on *Drosophila* behavior, and that I had finally "seen the light." I told him that I thought the best and most fun way to proceed would be to collaborate with him to follow up on his anecdotal observation suggesting that shocked flies emit a "Schreckstoff"–like substance that "scares" other flies. Expecting Seymour to be thrilled that I was finally going to take his advice and work with him on flies, I awaited his response.

There was a long pause at the other end of the phone line.

"No," Seymour said.

"What?!" I exclaimed. I couldn't believe what I was hearing. "What do you mean, 'no'?"

"No," repeated Seymour, simply and evenly, without further explanation.

I hung up, dumbfounded and disappointed. Here was Seymour, who had personally recruited me as a faculty member to Caltech, who had been encouraging me for years to work on *Drosophila*, giving me the brush-off after I had finally screwed up the courage to start working on flies--and in collaboration with him, no less! I simply couldn't understand his

negative reaction. So I called Larry Zipursky, Seymour's former postdoc. When I told him the story, Larry was not surprised.

"Seymour hates to collaborate," he said. I asked him why. Larry explained that Seymour was a solitary person, that he didn't like to depend on anyone else, that he had had bad experiences with politics in previous collaborations, and that he just wanted to be left alone to do his science. It was nothing personal, Larry said.

I was devastated, and so was Greg. That night, I thrashed fitfully in bed, unable to fall asleep. I worried that I had misled Greg into thinking he could work with the great Seymour Benzer, and now that door had been closed in our face. I felt angry and depressed.

That is, until the next morning when I arrived at the lab. Greg was already standing in front of my office and had a big smile on his face. He had stayed up all night to repeat Seymour's "Schreckstoff" experiment in secret, together with a French postdoc in Seymour's lab, Anne Simon. Greg showed me a bar graph with quantitative results from the experiment. It had worked, amazingly well. Seymour's observation was reproducible, robust and could form the basis of an exciting research project to search for the fly Schreckstoff and the brain circuits that sensed and transformed it into a fear response. But what to do, given that Seymour had said 'no' to my offer of collaboration?

At 3 PM (the time at which Seymour usually showed up for work, being a night-owl), the phone in my office rang. It was him.

"Well," he said without any pause, "it looks like we're in bed together." Just like that, all of a sudden, he had changed his mind and agreed to the collaboration.

I called Larry Zipursky. "I can't believe it," I said, "First Seymour says 'no,' then he gets one piece of positive data and flip-flops completely." Once again, Larry said that was typical Seymour – if he got an exciting result, he couldn't resist chasing it.

From that point on, Seymour did not simply embrace our collaboration – to the contrary, he virtually took over the entire project. He gave Greg a bench in his lab and insisted that most of the experiments be carried out there. As a result, Greg was hardly ever in my lab. In essence, Greg became Seymour's postdoc, working on Seymour's project – except that Seymour still expected me to pay 100% of Greg's salary!

"Classic Seymour behavior," said Larry when I told him about the arrangement.

And that was the beginning of my research collaboration with Seymour. Several years later, in September of 2004, Seymour, Richard Axel and I published a paper together in *Nature* with Greg and Anne Simon as co-first authors[43]. Richard, Seymour and I were co-senior authors (with myself listed last since I had instigated the project), but we included a note at the end of the paper that said "R.A., S.B. and D.J.A. made equally minimal contributions to this work." The editor at *Nature* asked us if we were sure we wanted to include that statement, and we confirmed that we did. Years later at an HHMI meeting, Yuh Nung Jan (see Volume 8) showed it on a slide and recommended that all PIs indicate their contributions to their papers in this way.

The paper by Suh, Simon et al. demonstrated that shocked flies emitted CO_2 together with another substance which we called "*Drosophila* stress odorant" (dSO), which caused other flies

to flee. With help from Leslie Vosshall at Rockefeller (another former Axel lab postdoc), we showed that the CO_2 receptor neurons she had identified recently were necessary for that "fear"-like avoidance response, and that those neurons were themselves activated by dSO collected from freaked-out flies. It was one of the early studies to use genetic tools to conduct a mini-screen for Gal4 drivers based on behavioral assay, and 2-photon calcium imaging to identify a circuit mediating an innate behavior in flies. A month after the paper was published, Axel and Linda Buck shared the 2004 Nobel Prize for their discovery of odorant receptors.

Three years later, Seymour passed away at the age of 86. I co-wrote his obituary for *Nature* with Sydney Brenner. I feel extremely fortunate, not only to have known Seymour personally, but to have been able to work closely with him on a collaborative research project. It wasn't easy – Seymour could be a difficult and quirky person – but I learned as much about the man as I did about his science. As Jonathan Weiner's biography "*Time, Love and Memory*" shows so beautifully, Seymour was a true original: a scientific pioneer and iconoclast who could see to the heart of any scientific problem and think of a creative way to solve it. I am grateful to have had the chance to see such a great mind at work up close and personal – even if at times he drove me crazy.

In the winter of 2006, I took a sabbatical with Martin Heisenberg (whom I had met on his own sabbatical at Caltech) to learn how to work with fruit flies and study their behavior. Martin was then Director of the Biozentrum at the University of Wuerzburg in Germany. He gave me an empty lab bench in the basement, and graciously made all the facilities of the institute available to me – including the expertise of the brilliant mechanical engineer Hans Kadershabek, who could cut plexiglass with millimeter precision and who made me a variety of gadgets I designed for experiments to test whether flies have an internal defensive state analogous to "fear." (In the modern era of CNC milling machines, this kind of expertise has sadly become a lost art.)

I had a wonderful time – it reinvigorated me intellectually, I fell in love with fruit flies and discovered that I actually had a talent for improvising prototype behavioral set-ups using discarded pieces of styrofoam and carboard boxes that littered my abandoned lab. (That may have been the only gene I inherited from my father.) However, try as I might, I could not convince myself that the innate escape responses to various aversive stimuli exhibited by my flies were anything more than glorified reflexes. For every behavior I observed that superficially resembled an "emotional" response (such as hiding under a barrier from an overhead threat stimulus), it was difficult to design experiments to rigorously exclude the alternative hypothesis that it was a sophisticated but robotic response (which I called the "Dickinsonian Hypothesis).

About the only thing I accomplished in Wuerzburg of any value was to perform the first successful optogenetic behavioral experiment in an adult fly. One day Georg Nagel, who had cloned the gene encoding Channelrhodopsin-2 (ChR2) together with Peter Hegemann, was visiting the Biozentrum to see whether expressing ChR2 in fly larval motor neurons would cause time-locked responses to blue light (which it did). I got the (derivative) idea to express it in he CO_2-sensitive Gr21a olfactory sensory neurons that Leslie Vosshall had recently identified[44], (which we had shown in the 2004 paper with Axel and Benzer were necessary for CO_2 avoidance) and see if it could confer behavioral avoidance of blue light (which flies are normally attracted to). Since Nagel was a generous person (he had shared his cloned ChR2 gene with Karl

Deisseroth but has received none of the credit for optogenetics), I asked him for permission to try the experiment--and it worked! Eventually, we published that result in 2007[45], a paper quickly forgotten because very few people could get ChR2 to work reliably in the adult fly CNS due to the inefficient cuticular penetration of blue light. (We were lucky to have chosen an olfactory neuron to test optogenetics in adult flies, because the cells are located directly under the antennal cuticle, allowing adequate blue light penetration.) It wasn't until 2013-4, when independently Ed Boyden's and my lab (in collaboration with the late Roger Tsien) demonstrated that red-shifted opsins such as ReaChR and Chrimson could be used for deep-brain optogenetic stimulation in adult flies[46, 47], that optogenetics in *Drosophila* finally took off – almost a decade after its use in mammalian neurons was first demonstrated by Boyden and Deisseroth[48].

From fear to fighting

I returned to Caltech from Wuerzburg in 2006 determined to see if I could rigorously demonstrate the existence of a fear-like state in *Drosophila*. Although I eventually provided evidence of a persistent, scalable and negatively valenced internal defensive state in flies[49], I became increasingly disenchanted with using fear as a model system to study analogous emotion states in flies and mice, for two reasons. First the standard behavior used to measure fear in mice was freezing, and at the time there was little to no rigorous evidence of freezing in flies evoked by threatening stimuli (although we eventually identified it in Gibson et al). Second, our studies of conditioned fear in mice targeting central amygdala PKC-δ^+ neurons[50] had failed to yield clear and robust behavioral responses to optogenetic stimulation, perhaps reflecting complex reciprocal inhibitory circuitry between these and other CeA GABAergic subpopulations.

During my sabbatical in Martin Heisenberg's lab there was a student (Suse Hoyer) working on aggression in flies, the study of which had been reinvigorated by Ed Kravitz (see Volume 4) at Harvard in 2002. It was obvious to even a lay observer like me that flies, with their robust lunging, tussling and boxing behavior, were engaging in aggression. That overcame the challenge of convincing people that flies had a fear-like state, when they didn't exhibit freezing like mice. Clearly, they exhibited aggressive behavior analogous to that performed by mice. So in 2006 I decided to start winding down our work on fear, and build a platform to study aggression circuitry in flies and in mice.

What did we want to understand about aggression, and why try to study it in two different organisms separated by 500 million years of evolution? Wasn't doing it on one critter plenty enough work by itself? A simple answer would be "Yes!" But further consideration (as usually happens when I overthink things) might provide some justification for this seemingly suicidal strategy.

The first challenge was to convince people that the problem of aggression hadn't already been solved. In 1943, the Swiss neurophysiologist Walter Rudolph Hess (not related to the other Rudolph Hess, who was unfortunately running an infamous concentration camp in Poland at the time), won the Nobel Prize for his experiments showing that electrically stimulating the medial hypothalamus in cats could transform a placid, purring pussy into a ferocious feline: spitting, hissing, teeth bared, tail puffed, back arched and ears laid back, as if another cat or a dog had foolishly wandered into its backyard territory. He called this response *Der affecktiven Abwehr reaktion* (the "defensive rage" reaction)[51]. Stimulation of other nearby sites evoked

different reactions, such as predatory aggression (hunting behavior). Hess's seminal experiment showed that an emotional (or "affective") state (as identified by its behavioral expression) could be evoked by electrical stimulation of a specific region of the brain. Therefore, the neural control of emotion states was not diffusely distributed over the entire brain, as commonly thought; rather it was localized in specific brain regions and therefore under regional control like other brain functions.

So, problem solved, Nobel awarded, case closed. What's left to do? (Indeed, after a conversation where I was describing our aggression results to Richard Axel, he quipped "Walter Hess says 'Keep up the good work!'" How's *that* for encouragement from a former mentor?).

Like any great experiment, Hess's results raised more questions than they answered. Were there really neurons in the brain that were dedicated to the control of aggression? If not, what other behaviors did they control? Were these neurons required for normal aggression, or was Hess's spectacular finding an artifact of strong electrical stimulation? (Efforts to answer that question during Hess's time using lesions had led to mixed results, generating controversy in the field). What did these neurons encode: a pre-motor program for defensive aggression; or some kind of internal emotion state akin to anger? What controlled these neurons and kept this potentially lethal behavior under wraps, until its expression was essential for survival?

To answer those and other questions, it was first necessary to identify the actual neurons responsible for brain-stimulated aggression. Having trained as a biochemist and molecular biologist, this was an obvious pre-requisite to me -- like having to purify an enzyme before you could properly study its function. (Apparently, however, it was not so obvious to some neuroscientists, like the reviewers who tried to reject our paper demonstrating optogenetic activation of aggression in mice on the grounds that we were just confirming Hess's results with fancy modern tools.) To do this, it made sense to pursue the problem in both mice and flies, because they afforded different and complementary experimental strategies. Since mice had the same basic brain anatomy as cats, rats, hamsters and other vertebrate species in which brain-stimulated aggression had been demonstrated over the years, we could exploit that knowledge (or "priors," as my Bayesian-minded friends like to call it) and apply it to the only mammalian organism in which genetically based approaches could be used to identify and functionally manipulate different neuronal cell types. By contrast in flies, where few if any such priors were available, we could take an unbiased, assumption-free brain-wide cell type-specific activation approach (analogous to a Benzerian forward genetic screen), to identify specific neuron that controlled this behavior – an approach that was impossible to apply in mice. Finally (and perhaps most importantly), pursuing the problem in two model organisms would (hopefully) allow us to identify potential evolutionarily conserved components of the aggression program, thereby distilling core features of its neural implementation from the baroque particulars of its circuitry in each species.

The flies have it

At a meeting in Beijing in the mid-2000s, I was approached by a young student named Liming Wang, who had taken an overnight train from Shanghai (an 8 hr ride) just to hear my presentation. He had done some undergraduate research on fly aggression, and immediately impressed me as bright, curious and ambitious, so much so that I invited him to join the

exclusive “speakers only” lunch and spent the entire time talking with him (to the evident annoyance of my not-so-egalitarian-after-all Communist hosts). I offered him a job in my lab as a research assistant on the spot. A few months later, he arrived in Pasadena with little more than a change of clothes in his suitcase and proceeded to set up fly fighting in my lab. A year later he applied and was accepted to the Biology Option at Caltech and continued as my graduate student. He published four major papers from my lab on fly aggression, including a cover article in *Nature* identifying the first aggression-promoting pheromone in *Drosophila*[52], and eventually graduated with the Ferguson Prize for best PhD thesis in Biology. He was among the best PhD students I have mentored in 40 years at Caltech – and I have been fortunate to have had many.

To realize my dream of a high-throughput behavioral screen in *Drosophila*, it was essential to develop a method for automated measurement of aggression in flies. It would simply not be feasible to screen thousands of pairs of flies for what might be subtle changes in aggressiveness by manually scoring videorecordings; the project would take years and require dozens of annotators. At that time (ca. 2007) the use of computer vision to measure objectively animal behavior was in its infancy, although Martin Heisenberg had taken important steps towards this goal[53]. To improve on this seminal work, I reached out to Pietro Perona, an avuncular, cultured and patrician Caltech professor of Electrical Engineering from Northern Italy specializing in computer vision, and convinced him to give the project a shot with the help of a German postdoc I had recruited with a fellowship from the Alexander von Humboldt Foundation (i.e., was free for Pietro who ran his lab on a shoestring budget). Collaborating with Pietro could be frustrating at times (while brilliant, he had a laissez-faire approach to competitive science and a European work ethic, taking 2 months off every summer to hang out with his numerous cousins on the Adriatic coast). Despite this difference in scientific styles, we published our first paper together in 2008[54] and have been collaborators and close friends ever since, bantering and bickering with each other like an old married couple.

In 2009 Pietro and I published a paper in *Nature Methods* describing an automated classifier for tracking and measuring aggressive behavior and courtship song in *Drosophila*[55]. Using this classifier Eric Hoopfer, a postdoc in my lab, screened almost 3,000 lines of transgenic flies generated and maintained by Gerry Rubin at Janelia Farm. Each line expressed the yeast transcriptional activator Gal4 in a different, randomly marked subset of neurons which we conditionally activated using dTrpA1, a temperature sensitive cation channel (red light-based optogenetics had not yet been introduced in flies). For each line, Eric tested a dozen fly pairs to see whether activating the channel at 27ºC could evoke fighting in otherwise passive flies made non-aggressive by group housing. It was the first “gain-of-function” cellular screen of its kind.

The next step was to pick one or two cellular “hits” and focus on them in detail to understand how they controlled aggression – analogous to “working up” hits in a genetic screen. In retrospect, we (I) could (and should) have made life easier for ourselves (i.e., Eric) by focusing initially on the line that showed the most robust and aggression-specific phenotype. However, given my inherent inclination to over-complexify things (indeed Mel Simon, a former chair of the Caltech Biology Division, once asked me whether I had Hungarian ancestry, since Hungarians apparently have a reputation for such complexification), I pushed Eric to focus on one line that exhibited an interesting and surprising phenotype: not only did activation of these neurons enhance fighting, but it also enhanced male-male courtship! This was bizarre, since

ordinarily aggression and mating are considered as opponent, mutually antagonistic behaviors. But I was motivated to focus on these cells because we had recently obtained apparently analogous results in our studies of mouse aggression neurons in the hypothalamus (see below), and I was eager to find homologous or analogous circuitry for aggression in the two species.

Remarkably, these seemingly dual fighting/mating neurons turned out to be members of a small, male-specific cluster of cells called "P1," which Barry Dickson had independently identified in 2011 using an unbiased neuronal silencing screen for neurons controlling male courtship[56]. In the end, we found that P1 neurons control a persistent internal state of social arousal common to both aggression and courtship[46, 57]. This was very gratifying, because our initial hope had been to identify not simply neurons that controlled various aggressive motor actions, but rather internal states underlying this behavior. P1 neurons have since attracted intense interest and become one of the most intensively studied cells in the field of *Drosophila* social behavior circuitry, for example in beautiful work by (former Axel lab postdoc) Vanessa Ruta at Rockefeller[58]. Ruta's work on P1 neurons has now far outpaced our own. I guess I can console myself that imitation is the sincerest form of flattery.

The mouse that roared

In 2009, I recruited a postdoctoral fellow to my group named Dayu Lin, who had trained with the late Lawrence Katz at Duke and had a background in electrophysiology, to begin our studies of aggression in mice. Her first step was to repeat Hess's basic experiment in mice, i.e., to electrically stimulate the ventromedial hypothalamus (VMH) and evoke attack. This seemed straightforward, since similar experiments had been reported in rats, guinea pigs and hamsters. Frustratingly, however, Dayu was not able to evoke attack, despite close to fifty attempts. Instead of attacking during the electrical stimulation, the mice became apparently fearful and froze or tried to jump out of their cage. Eventually we realized why, in the 80 years since Hess's Nobel-winning paper, there had been no published report demonstrating brain-stimulated aggression in mice: it's because the experiment doesn't work in that species.

Now we were really stuck. We wanted to study aggression using sophisticated new tools like optogenetics to identify the precise neurons involved, but those genetic tools could only be applied easily in mice--and Hess's experiment didn't seem to work in mice! What to do? Keep on trying and hope eventually to hit the right spot? Spend years re-developing from scratch all the tools for genetic manipulation of mice in rats, where Hess's experiment works well? Or quit and study a behavior that it is easier to get mice to do, like eating cheese?

Although Dayu was stimulating the ventrolateral subdivision of VMH (VMHvl), where c-fos -expressing cells were abundant following aggression, the fact that electrical stimulation evoked what appeared to be intense fear suggested to me that perhaps the current was spreading into the dorso-medial region of VMH (VMHdm), where cells controlling innate fear were thought to be located. And perhaps fear trumped aggression when artificially evoked (our later experiments showed this conjecture to be correct). Maybe this was a special problem in mice but not in rats because VMH was much larger in the latter species, so injected current couldn't spread as far into VMHdm as it could in mice.

At the time, Karl Deisseroth had recently shown that optogenetic stimulation in vivo could be achieved by injecting an AAV vector encoding ChR2 into the brain and delivering blue light to the injected region using an implanted fiber-optic cable. I reasoned that perhaps optogenetics could solve our problem, because the ChR2-expressing virus particle was huge in comparison to electrons, and therefore much less likely to spread by diffusion from VMHvl into VMHdm.

While we had no experience with optogenetics at the time, I was excited and enthusiastic about trying the experiment and offered to send Dayu up to Stanford to learn the technique from Karl. Dayu, however, thought it would be a waste of time. To her, optogenetics was just a different way to inject electrical current into neurons. Since she had already shown that approach didn't work, there was no reason to think optogenetics would work any better. Why should she spend months learning to master a new and untested technique, when the results wouldn't be any different from what she'd already obtained?

I begged and pleaded with Dayu, but she was stubborn and I couldn't convince her. In a last-ditch effort I tried one final argument, as we were walking together across the Caltech campus to have lunch at the Athenaeum, the venerable Spanish colonial-style faculty club where Einstein always stayed when he visited. "Dayu," I said, "if you don't try this experiment, and in another 6 months you pick up a copy of *Nature* and see a paper from another lab on optogenetic induction of aggression in mice, you will never forgive yourself." She paused, thought about it, and reluctantly agreed to try the experiment.

A couple of months later, in one of those moments that gets burned into your brain forever, I was sitting in my office when Dayu suddenly poked her head in the door, her eyes shining with excitement and a huge smile on her face. "It worked!" she said. "N equals one -- but it worked!" In the grainy video she showed me on her laptop, a mouse with a flexible fiber optic cable sticking out of its head tentatively approached a female mouse that had just been introduced into the cage. It sniffed, attempted to mount her, and then walked away. The next time the mouse approached the female, Dayu fired a blue laser, sending twenty short pulses of light per second cascading down the fiber optic cable to illuminate the ChR2-expressing neurons in VMHvl. Amazingly, instead of attempting to mount the female, after a few sniffs the male mouse lunged at her neck and nipped at her, as if she were a male intruder. After a brief tussle, Dayu switched off the laser. The attacking mouse nipped at the female a couple of more times, then stopped and walked away from her. The next time the mouse approached, with the laser off, it interacted with the female normally. And then, with the laser switched on, it attacked again, until the laser was turned off. We had literally triggered attack with the flip of a switch.

"N=1: out of 9 mice in which Dayu initially tried the experiment, only one exhibited aggression when the light was switched on. But that one mouse was enough to show us that there must be aggression-promoting neurons in that tiny region of the brain, after all. And in a moment, it changed everything. Eventually Dayu was able to refine her injection coordinates to be able to evoke aggression reproducibly in multiple mice. In 2011, we reported the result in an article in *Nature*[59] together with evidence that silencing cells in VMHvl inhibited aggression, using an early implementation of chemogenetics based on expression of a Ivermectin-activated chloride channel developed by my colleague Henry Lester at Caltech[60]. We may only have succeeded in repeating the Hess experiment with fancier tools, as one of our negative reviewers

had snidely argued. But we had introduced optogenetics into the study of social behavior for the very first time, and more generally had established the mouse as a system where genetically based tools could be used to probe neural circuits underlying social behaviors. This is now a large and thriving field led by my scientific children and grandchildren.

Like my first paper on the in vitro synthesis of the AChR, the 2011 *Nature* paper was perhaps more significant for what it portended about the study of social behavior, than for any specific finding or conceptual advance it contained. But it opened a new field of research, and from that perspective feels like one of the most important papers I have ever published. The problem was that we had no marker for these aggression-promoting cells at the time, hampering further progress. Eventually, in 2014 we were able to genetically identify the VMHvl neurons sufficient to promote aggression, by making transgenic mice which restricted ChR2 to type-1 Estrogen Receptor-expressing neurons[61]. While my former PhD student Nirao Shah at UCSF (also an ex-Axel postdoc) partially scooped us by reporting in 2013 that genetic ablation of Progesterone Receptor-expressing VMHvl neurons (which overlapped $Esr1^{+}$ cells) inhibited aggression[62], it fell short of our goal of identifying the neurons that mediated brain-stimulated aggression (BSA)--the phenomenon discovered by Hess almost a century earlier[63]. (While Nirao's data showed that $VMHvl^{PR}$ neurons were necessary for attack, they did not exclude a purely permissive function for the cells, analogous to gas in a car. For example, the mouse's nose is also necessary for aggression, but that doesn't mean that stimulating the nose should necessarily elicit attack.) Nevertheless, the episode still felt a bit like scientific "patricide," as Günter used to call it.

In the end, did we discover any interesting homologies or analogies between flies and mice in the neural control of social behavior? As described above, our unbiased screen for aggression-promoting neurons in flies identified cells (P1 neurons) able to promote both mating and fighting[57]. Similarly, we found, surprisingly, that optogenetic activation of $VMHvl^{Esr1}$ neurons could promote either mounting or fighting in mice, depending on the stimulation intensity[61]. At first, I thought this might reflect an evolutionarily conserved circuit motif for multiplexed neuronal control of sex and violence[64]. Later, however, we discovered that the male-directed mounting promoted by weak $VMHvl^{Esr1}$ activation was a low-intensity form of aggression (i.e., dominance mounting), rather than sexual behavior[65]. This seemed to throw cold water on the idea that the same cells control mating and fighting in both species. Later experiments, however, confirmed that $VMHvl^{Esr1}$ neurons *as a population* indeed play a role in male-female mating[62] as well as in aggression, but that the individual cells controlling these behaviors are largely distinct[66-68]. Similarly, recent evidence suggests that distinct subtypes of P1 neurons promote courtship vs aggression in flies[69, 70], a possibility we had previously acknowledged[64]. Together, these data suggest that P1 neurons in flies and $VMHvl^{Esr1}$ neurons in mice play roles in both mating and aggression not because the same *individual* neurons control both behaviors, but because within the same tiny region of the brain, distinct subsets of molecularly similar but anatomically neighboring cells control these social behaviors. Whatever the granularity of the explanation, the fact remains that the neurons controlling sex and violence are inextricably intertwined in the brain, a finding with potential implications for psychiatry[71].

Coda: Towards a systems neuroscience of the hypothalamus

I don't want to leave you with the impression that the control of aggression can be reduced to a handful of cells in a single nucleus of the hypothalamus. Indeed, recent studies by my former postdoc Dayu Lin and others have identified a meso-scale network of interconnected hypothalamic nuclei (and other subcortical structures) that promote or inhibit aggression, at least in part through their influences on VMHvl[72]. In addition, recent work led by my postdoc Ann Kennedy and student Adi Nair has revealed that even within VMHvl, internal states of aggressiveness may be encoded not simply by the activation of a specific neuronal subtype, but rather by population-level neural dynamics that produce emergent properties such as line attractors[73] (or "leaky" integrators)[74]. Importantly, these dynamics are not a *consequence* of aggressive behavior, because they can be observed in head-fixed animals passively observing two other mice fighting[75], among cells that have been suggested to "mirror" aggression[76]. These data suggest that such attractor or integrator dynamics may compute a variable(s) encoding the intensity and/or duration of an aggressive internal state[74]. We have also observed similar population dynamics underlying female mating receptivity[77]. These and other data[78, 79] suggest that such mechanisms may be used to encode multiple types of internal states[80]. "Manifold-level" mechanisms[81] have been well-established in cognitive neuroscience[82-85], but the idea that they may also play a role in representing homeostatic or affective internal states is new. The implementation, read-out and causal role of these mechanisms remain to be established.

Clearly there is much to be done to understand how internal affective states such as aggressiveness are implemented across different physical and temporal scales in the brain and the body. Eventually, I hope we will be able to understand what is really going on in someone's mind when they become angry, enraged or furious. Such an understanding might someday help to reduce the chronic violence that afflicts our society[86].

Concluding thoughts

People often ask me why I've changed fields so many times. It's true: over the last ~50 years I've worked in cell biology (membrane protein biogenesis), molecular biology (transcriptional control of gene expression), developmental biology (neural crest stem and progenitor cells), sensory neuroscience (cutaneous polymodal nociceptors and gentle stroke-sensing mechanoreceptors), behavioral neuroscience (neural circuits controlling affective states), with a major digression into angiogenesis (the accidental discovery that arteries and veins are genetically distinct before the heart starts to beat; awkwardly my most highly cited paper). I've worked on rats, mice, fruit flies and even jellyfish[87]. As the late David Baltimore somewhat sardonically remarked: "I don't think there's an area of biology that you haven't touched."

The answer to this question has many levels. First and foremost, I enjoy the excitement of opening up a new field. The feeling that you are doing something that nobody else is crazy enough to try (like imaging neural activity in baby jellyfish) is both exhilarating and liberating. Once the initial excitement dissipates, however, I find it hard to maintain the level of inspiration and creativity necessary to identify the most conceptually important and tractable next experiments, and my science becomes more incremental and pedestrian. Third, I dislike working in highly competitive fields, so once the next experiments become too obvious, I feel it's time for me to move on and leave the field to other investigators who are more organized

and efficient than I. Although I've tried to maintain a focus on aggression circuitry for close to 20 years, now even that once-obscure field has also become too competitive (thanks in large part to the beautiful work of my more capable scientific progeny) and I'm moving on to computational neuroscience[74]. The learning curve has been steep, but that's the fun part.

Individual papers notwithstanding, I feel that my most impactful contribution to neuroscience has been to convince the late billionaire philanthropist Paul Allen in 2002 to focus his new brain institute on generating a publicly accessible, comprehensive atlas of gene expression in the mouse brain, as its inaugural project[88]. The resulting Allen Brain Atlas[89] was (and continues to be) a useful resource for many neuroscientists and has spawned a whole series of high-quality atlases generated by the Allen Institute of Brain Science (AIBS) at different scales (axonal projections, cell types, enhancers, etc.) and in human as well as mouse brains, under the capable leadership of first Allan Jones and Christof Koch and now Hongkui Zeng, Ed Lein and President Rui Costa. It's not something I could ever have accomplished myself, but the completion of the first searchable *in situ* hybridization atlas of all the genes expressed in the murine brain gave me the immense satisfaction of watching a whole team of talented scientists and engineers turn a vision I had into a reality.

I've always enjoyed science as a social activity and owe a great debt of gratitude to my many colleagues, friends and mentors over the years, including Ralph Adolphs, Richard Axel, David Baltimore, Günter Blobel, Cori Bargmann, Seymour Benzer, Norman Davidson, Gerry Fischbach, Ed Furshpan, Ulrike Heberlein, Martin Heisenberg, Eric Kandel, Ed Kravitz, Menno Kruk, Story Landis, Scott Linderman, Paul Patterson, Pietro Perona, Martin Raff, Gerry Rubin, Josh Sanes, Mark Schnitzer, Mel Simon, Charles Stevens, Doris Tsao, Irving Weissman, Barbara Wold, Hongkui Zeng, Kai Zinn and Larry Zipursky. They have provided much-needed wisdom, help and advice as well as the joy of intellectual stimulation and interaction.

Last, but definitely not least, I'd like to thank all of the students and postdocs who have contributed to the work in my lab, many of whom have gone on to their own illustrious careers. Richard Axel once said to me: "You're only as good as your last paper." Likewise, I'm only as good as the best person in my lab at any given time. Fortunately, over the last 40 years I've been lucky enough to surround myself with people smarter and more capable than myself as I sought the road less traveled. And that, as Robert Frost said, has made all the difference.

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