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Joseph S. Takahashi

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  December 16, 1951

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  University of Texas Austin, Zoology (1975–1979)
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  Fellow, Pharmacology Research Associate Training Program, NIH/NIMH (1981–1983)
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  Honma Prize in Biological Rhythms Research (1986)
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  W. Alden Spencer Award in Neural Science, College of Physicians and Surgeons, Columbia
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  Eduard Buchner Prize, German Society for Biochemistry and Molecular Biology (2003)
  Member, National Academy of Sciences, USA (2003)
  Member, Academy of Medicine, Engineering and Science of Texas (2009)
  Outstanding Scientific Achievement Award, Sleep Research Society (2012)
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Joseph Takahashi is a pioneer in elucidating the genetic and molecular mechanisms of the mammalian circadian clock system. His lab discovered the first circadian clock gene in mice and humans, named "Clock." The CLOCK protein and its heterodimeric partner, BMAL1, act as the primary transcriptional activators of the circadian gene network in animals. He used mutagenesis and behavioral screening in mice to isolate the Clock mutant mouse, which then opened the way to find the underlying gene by positional cloning and transgenic rescue of circadian rhythms at the behavioral level. Many years later, his lab was able to solve the crystal structure of the CLOCK:BMAL1 complex; thus, going from a genetic screen for a mutant mouse to the underlying gene and protein, and the solution of its atomic structure. Before the discovery of clock genes in mammals, the significance of circadian rhythms to essential cellular functions was unclear. With the discovery of the clock gene network and the finding that CLOCK:BMAL1 are upstream regulators of thousands of genes in cells, molecular interactions have revealed that the circadian clock system directly regulates metabolism, immune function, cell growth and cancer, sleep, neurological diseases, aging, and longevity.

Joseph S. Takahashi

My Family

I was born on December 16, 1951, in Tokyo, Japan. My parents met in Tokyo as a consequence of the postwar occupation of Japan by the U.S. government following the end of World War II (WWII). My father, Shigeharu Takahashi (1921–2000), worked as a civilian agricultural economist in General Douglas MacArthur's staff in Tokyo. He obtained that position by virtue of being in the U.S. Army, being a Japanese-American who could speak both English and Japanese, and having graduate training in agricultural economics. My paternal grandfather, Shigetaro Takahashi (1877–1955), immigrated to the United States in 1896. He was the second eldest son of a rice-farming family in Wakayama prefecture in Japan. Because the eldest son inherits the rice farm, my grandfather had to find something else. He decided to go to San Francisco and there he established a successful "Oriental" import business named the Kisen Company Silk House on 157–159 Geary Street about a half block away from Union Square. During the 1906 earthquake and fire in San Francisco, the Kisen Company store sustained damage but survived and continued until about 1920. My paternal grandmother, Hana Morikawa (1884–1957), married my grandfather in 1910. They had four children: Kiyo (1913–2015), Shigeharu, Tomiko (1918–2011), and Edwin (1929–2009). They lived on Pine Street in San Francisco, and for most of their lives, their home was a safe harbor for Japanese immigrants trying to find their way in the United States. During the Depression, my grandfather was able to take over a store that had gone out of business and opened the second Kisen Company store on 456 Grant Avenue from about 1930 to 1942, when they had to vacate it and sell off the inventory at a huge loss.

After the attack on Pearl Harbor, about 120,000 people of Japanese ancestry were forcibly relocated and incarcerated in concentration camps in the western United States under Roosevelt's Executive Order No. 9066. My father was the only member of our extended family who was not interned (with the exception of an aunt and two cousins who were visiting relatives in Japan when the war broke out). As described by my cousin, Ruth Sasaki,[1] our family in San Francisco was taken to the Tanforan "Assembly Center" in San Bruno (a barely repurposed race track) for about six months; and, then they were transferred to the Topaz Relocation Center in Utah in September 1942 until around September/October 1945, when the camp closed. They lost most of their property (real estate) and possessions and had to sell them for less than 10 cents on the dollar.[2] My aunt Kiyo worked in medical records at the Topaz Hospital before she resettled in Chicago in 1944, and my aunt Tomi was the Supervisor of Topaz preschools. After release from

the "camps" in 1944–1945, Japanese Americans continued to face discrimination in housing, violence, vandalism, and defacing of Japanese graves. Only many decades later was it acknowledged in 1976 by President Gerald Ford's Proclamation 4417 that the internment was "wrong" and "that this kind of action shall never again be repeated"[3] and by a Congressional report in 1983 that "Executive Order 1066 was not justified by military necessity, and the decisions that followed from it—exclusion, detention, … were not founded upon military considerations. The broad historical causes that shaped these decisions were race prejudice, war hysteria and a failure of political leadership."[4]

In 1942, my father was a student at the University of California Berkeley. When the internment began in San Francisco, professors at Berkeley tried to get many of the nearly 700 Japanese American students transferred to the east so that they did not have to be interned in the relocation camps. My father and another student were transferred to Ohio University in Athens, Ohio. When they arrived at Ohio University, the administration reversed its decision and said sorry you cannot transfer. Then the most amazing thing happened: the student council and students protested the administration's reversal and eventually the administration backed down and admitted my father and the other student. Thus, my father was the only member of his extended family who was not interned during WWII. Eventually my father graduated from Ohio University and entered graduate school in economics at the University of Chicago from 1944 to 1945. Ironically, after a year and a half in graduate school, he was drafted into the U.S. Army. He was stationed at the Defense Language Institute Foreign Language Center in Monterey, CA, where he taught Japanese to U.S. troops, and he rose through the ranks to master sergeant. When the war ended, he was recruited to serve in the postwar occupation of Japan led by MacArthur.

My mother, Hiroko Hara (1920–1984), was born in Kobe, Japan. My maternal grandfather, Kumetaro Hara, and grandmother, Hisako Nakamura Hara, came from privileged families in Japan (descendants of the Seiwa Genji clan). My first cousin once removed, June Grandwells, was told the following family legend by her grandmother, Masa (who was Kumetaro's daughter-in-law):

> One of our ancestors in the Hara family was the Head Retainer of the Kishū House (Kishū is actually a name of the place, present Wakayama), one of the three branches of Tokugawa clan, established by Ieyasu to provide successors to the Tokugawa shogunate in case the main line should become extinct. As the fifth head of Kishū House became the eighth Shogun Yoshimune (Tokugawa), it is possible that the Head Retainer accompanied him to Edo (present Tokyo) Castle, the center of the shogunate, although I have absolutely no proof of our Head

Retainer serving in the Yoshimune's era.… Under the Peerage
Act of July 7, 1884, the Meiji government expanded the heredi-
tary peerage with the award of Kazoku (aristocrat) status to
persons regarded as having performed outstanding services to
the nation. The former 15th shogun, Tokugawa Yoshinobu,
became a prince (or a duke), the heads of primary Tokugawa
branch (shinpan Daimyo) became marquises, and the heads
of the secondary branches became counts. I was told that one
of the members of the Hara family was offered to be awarded
Kazoku status (possibly the count, the highest honor for anyone
from a non-Kuge or non-Daimyo house) for his achievement
from the Meiji Emperor in person. The person in question was
most likely your grandfather or possibly great-grandfather, who
unbelievably turned the offer down.

As a consequence of his family's standing, my maternal grandfather
went to college at the University of Pennsylvania in 1903. Then he obtained
his doctorate in engineering in Berlin and worked as a senior engineer at
Kawasaki Marine Engineering. Because of his experience in Europe, he
loved classical music and wore western clothing in Japan. They had six
children, the youngest of whom were my aunt, Chieko Hara (1914–2001),
and my mother. Both Chieko and my mother went to Sacred Heart School
in Tokyo (Seishin Joshi Gakuin) and were raised as Catholics. There they
both learned to speak English. They also were trained in piano, and my
aunt was sent to Paris at the age of thirteen, where she studied with Henri
Gil-Marchex (1894–1970) and Lazare Levy (1882–1964). In 1932, Chieko
won the *Premier Prix* at the *Conservatoire de Paris* becoming the first
Japanese pianist to enter and win this prize. She then had to return to
Japan, but subsequently returned to Paris to study with Alfred Cortot
(1877–1962). In 1937, Chieko went on to win a special prize at the Third
International Chopin Piano Competition in Warsaw, Poland, and became
an acclaimed concert pianist in Europe. The story about my aunt is too
involved to describe here, but Rie Ando wrote a wonderful doctoral thesis
(2010)[5] chronicling her artistic and tumultuous life.

Japan, Burma, and Pakistan

Although my mother's family was well off before WWII, they lost everything
during the war. During the postwar occupation, MacArthur's rebuilding of
Japan's economy involved many large contracts with Japanese companies. My
mother by virtue of having gone to Sacred Heart School could speak English
almost fluently and was able to get a job as a bill collector for one of these
companies doing business with the U.S. occupation forces. My father's boss in
MacArthur's staff was John Cooper, and he had met my aunt Chieko at one

of her concerts in Tokyo and found out that she had an unmarried sister. He told my dad that he should meet her and made the introduction. This is how my father and mother met each other in Tokyo. Initially, American occupation forces were not allowed to marry Japanese "aliens," but eventually this prohibition was lifted, and my parents were married in November 1950. The following year, I was born in a U.S. Army hospital in Tokyo. The occupation of Japan formally ended on April 28, 1952, and at that time, my dad found a new job as an agricultural economist with the Robert Nathan Associates firm in Washington, D.C. We made our way to Washington when I was a baby, and then my father was put on foreign assignment in Burma (Myanmar). We moved to Burma in 1952 and lived in Rangoon, Burma, until 1958 when I was seven years old. This was the beginning of the military control of Burma when General Ne Win was invited by Prime Minister U Nu to take over the country and later a series of military coup d'états led to the complete control of the Burmese government by the military for the next 60-plus years.

Burma was a lush tropical paradise and was the beginning of my experience and love of nature. My mother loved animals, and we had many pets (beloved Siamese cats and a Lhasa Apso dog). In addition, there were chickens, ducks, and more dogs owned by our house staff known as "domestic servants" in the day. This was a remnant of the British colonies in India of which Burma was a part before their independence in 1947. I had a nanny who was Karen, an ethnic group in southeastern Burma, and through her I learned to speak both Burmese and Karen fluently as a child. Sadly, after leaving Burma, I never spoke Burmese again, and I cannot remember a word. I always wonder whether those languages might still be lurking somewhere in my brain.

My father loved to fish, and we went on many fishing trips in Burma. There was a lake down the street from our house where we caught carp, catfish, and snakehead fish (called *nga yant* in Burmese). My father also took me on field trips up the Irrawady River into the jungle. We would have armed guards on board the boat because of the bandits further up the river. In these outings into the jungle, we would see many exotic birds, monkeys, snakes, and turtles, and of course, we caught many different types of fish. Green peacocks are the national symbol of Burma, and we would see these often. Elephants and water buffalos were the workhorses in Burma. Timber elephants were used for lumbering of teak and carrying heavy loads. Each elephant had a mahout (rider and trainer), and you could see the special bond between each pair. Water buffalo were used for plowing rice fields and their milk was used for many dairy products. I also remember all the unusual insects there: giant millipedes the size of a cigar, large black scorpions, huge grasshoppers, and crickets (that were fried and sold at markets). It was like living in a zoo!

Because of the military takeover of Burma in October 1958, we had to leave the country and my father had to find a new job and was engaged

in consulting work with USAID in South America. Meanwhile, my mother was pregnant with my sister, Nancy (1959–2012), and so my mother and I moved to Rome, Italy. My mother chose Rome for two reasons: she wanted to be close to her sister, Chieko, who now lived in Florence, Italy, and she wanted to deliver Nancy at the Seventh-Day Adventist Hospital in Rome. On May 9, 1959, my aunt Chieko married the Spanish cellist, Gaspar Cassado (1897–1966),[6] in Siena, Italy. The wedding took place in Count Chigi's (Guido Chigi Saracini, 1880–1965)[7] palace in Siena (Palazzo Chigi-Saracini) around the time of the Palio of Siena festival. Typical of Catholic weddings, it was a very long affair. I was a ring bearer in the wedding party, and there was great fanfare around the wedding (my first time in newsprint and TV). Count Chigi was a patron of the musical arts, and he founded the Academia Musicale Chigiana in 1932 and the Siena Musical Week Festival in 1939. Both Alfred Cortot[8] (Chieko Hara's teacher) and Pablo Casals[9] (Gaspar Cassado's teacher) attended the wedding. In Italy, it was customary for children to drink both coffee and wine at a young age by watering down the drinks to reduce their punch. So, coffee drinking began at the age of eight for me, and at the wedding, I was able to partake in champagne. I have photos of my mother looking at me from across the room with her mouth agape because I was happily quaffing champagne.

In Rome, our apartment on Via Veneto overlooked what was to become the Olympic grounds in 1960. Because my father was overseas, my mother had a wonderful chauffeur named Vincenzo. He would take my mother and me on an outing every weekend to museums, Roman ruins, gardens, and drives in the countryside. It was truly a cultural experience for a young child. Sadly, we only lived in Rome for one year. My father secured a job at the World Bank in Washington, D.C., and shortly afterward, we moved to the D.C. suburbs in Bethesda, Maryland. We lived about one mile away from the National Institutes of Health (NIH) campus. I went to Alta Vista elementary school from the second to fourth grade. Then my father was assigned to Karachi, Pakistan, in 1962. We lived in Karachi for two years during which I went to Karachi American School for fifth and sixth grades. Similar to my experience in Burma, Pakistan was a beautiful place, but instead of a jungle, it was an arid desert. Again, the animal life was exotic: camels were the primary beast of burden. I also had friends whose fathers took us hunting for deer and wild boar as well as ducks and doves. But, most memorable for me was fishing with my dad almost every weekend on the Arabian Sea. The Arabian Sea is like most tropical oceans and had abundant schools of crevalle jack, blue crevalle, king mackerel, mahi-mahi, bonito, barracuda, cobia, grouper, and red snapper. Schools of dolphins and porpoises were frequently seen, and many would surf on the bow wave of the fishing boat as we trolled along. We also had a friend of the family who had a cottage on the beach with a two-mile stretch of sand flanked by rocky reefs. There, we found all sorts of intertidal wildlife among the seaweed and rocky pools.

I also remember vividly being able to see giant sea turtles laying their eggs on the beach at night. All of these experiences forged my love of the ocean and aquatic organisms.

Perhaps most influential for me in sparking my interest in biology in Pakistan was the hobby of keeping pigeons as pets. I started with a pair of pigeons that we bought at the bazaar in Karachi. We built a coop on the roof of our house, which had a flat cement roof. When first bringing home a pigeon, they plucked the distal wing feathers so that the bird could not fly well until the feathers grew back after a few weeks. Then you gave the pigeons a nice home with food and water, and magically after that they stayed even though they could fly free. Gradually, I would go to the market and buy more pigeons. There were all kinds of "sport" pigeons in the market, including different coloring, fantails, and "tumblers," which were pigeons that did backflips when they were flying. Once you had a flock of pigeons, they would attract other pigeons that might fly by. Or if you saw a stray pigeon flying around, you would go to the roof and send your flock to fly in the air. The flock of pigeons typically would fly in circles around the rooftop before landing back home after a while. Most of the time, the stray pigeon would join the flock in flight and then eventually land on the roof. I would then leave a trail of bird seed from the roof into the pigeon coop. When the stray pigeon entered the coop, I would close the door and go into the coop and catch the pigeon. We then plucked its flight feathers, and it would stay on the roof until it could fly again, and by that time, it had found a new home. One of the most interesting things that the pigeons did was to court and form pair bonds. Then they would build nests in their nest boxes, lay eggs, and take turns incubating them (about 18 days), and pretty soon there would be hatchlings. The parents fed the hatchlings and squabs by regurgitation of crop milk. After about four to six weeks, the pigeons become independent and looked like adults. I was fascinated by the entire life cycle of pigeons and also by the progeny from the various mated pairs since many pairs had different coloring, tails, or behavior (tumblers). These were my first lessons in animal breeding and genetics. The inheritance of different colors of feathers was interesting, but perhaps most interesting was the inheritance of tumbling behavior, which appeared to be genetic rather than learned.[10]

In addition to the natural world in Pakistan, I was also exposed to the stark realities of the developing world. Not far from our home in Pakistan, there was a refugee camp behind a tall chain-link fence enclosure. The residents, I was told, were from India and made a living producing cow dung cakes that were used for fuel, resulting in an income of less than $200 per year in the 1960s. We frequently were accosted by beggars on the streets. Sadly, many of these individuals had been maimed on purpose to promote their life of begging—for example, by cutting off their tongue so that they could not speak. Others were blind or were lame and could not do normal tasks. In the bazaars, one would be constantly pursued by young children begging for

money. I had to learn at an early age to steel myself from these sad souls and try to ignore their calls. Such poverty and dire living situations were very eye-opening for me and made me greatly appreciate life in the United States.

President John F. Kennedy was assassinated while we lived in Pakistan, and I remember that day vividly. My friend whose father was in the military called me to tell me about Kennedy. I was a great fan of JFK and had a large poster photo of him in my bedroom. We were all devastated by his senseless loss.

Bethesda and Rockville

In 1964, we moved back to Bethesda, Maryland, and I went to North Bethesda Junior High School for seventh and eighth grades. I took up playing the guitar and took classical guitar lessons and also was a rhythm guitarist in a rock and roll band. We played a lot of gigs and one of the cadre of musician friends back then was Nils Lofgren.[11] Nils was amazing. He picked up the guitar and in months he was already professional! My friend Tom Miller was in a band with Nils, and one time, our band was in a battle of the bands at the junior high with Tom and Nils's band. They beat us handily.

One of my hobbies during junior high was raising tropical fish. I was especially fascinated with cichlids, angel fish, and discus. These fish had elaborate mating rituals and would lay eggs and care for them. To provide conditions under which the fish would breed, I had to learn about their native habitats and create appropriate conditions for them. For example, discus were the most difficult to breed, and the water had to be extremely soft and treated with peat to mimic the acidic water of the Amazon. My other hobby at the time was tinkering with engines and go-karts, which I loved to drive at the Korvettes shopping center parking lot in Rockville on Sundays when the stores were closed. I loved taking apart the engines and putting them back together (without too many parts leftover).

During this time, my parents bought a property in Potomac Highlands, which is between Rockville and Potomac, Maryland. They designed and built a new house on the property and after eighth grade, we moved to Rockville. I went to Julius West Junior High School for nineth grade, and then Richard Montgomery High School in Rockville. At the time, Richard Montgomery was 50 percent college prep and 50 percent vocational. (Today Richard Montgomery is a magnet school.) Because it had a vocational school, there were many practical courses that I could take as electives. So, I was able to take mechanical drawing, industrial arts (shop), and my favorite, auto mechanics every year. For our senior year independent project, my best friend, Walter Gardiner, and I rebuilt the V8 engine in his 1959 Ford Thunderbird. These vocational courses turned out to be extremely helpful many years later. Mechanical drawing made it easy for me to read and understand architectural plans for lab renovations and new buildings; shop honed

my woodworking and machine shop skills; and auto mechanics taught me much about how things worked and how to repair them. All useful skills in life and in science. I found academics relatively easy in high school, and as a consequence, I did not have to study very hard or develop good study habits. Despite this, I graduated third in my class. I was also vice president of the Student Council and Mike McCord was president. We had meetings at his home in Rockville, and I met his father, James W. McCord Jr., who would become infamous as one of the Watergate burglars a few years later in 1972.

As soon as my friends and I turned sixteen, we were able to get jobs and work during the summers. We mostly started with jobs at the local country club nearby. We began as dishwashers and then could be elevated to busboys. In my case, I was promoted to an assistant cook in the main kitchen (for reasons that I don't remember or fathom). There was the executive chef of the kitchen and a second chef, and the assistant cooks. It was a very interesting learning experience for me to prepare meals on a large scale and to use all of the elaborate kitchen equipment. As the summer progressed, I was given more responsibility and autonomy, and I learned to plan and execute meals to be prepared at specific times. My mother taught me how to cook and, as a child, I had learned to prepare my own breakfasts before school, so she prepared me for my summer job as a cook. Another funny episode at the country club was an annual event called the Hawaiian Luau. My additional task as assistant cook was to dress up in a Hawaiian costume and turn the roasted pig on the patio, likely because I was the only Asian working there (but I didn't mind because I love Hawaii). The next summer, I was promoted again to become the cook for the Men's Grill (very sexist back then!). This was a small kitchen and bar that was open for breakfast and lunch. There were only two of us: Larry the bartender, my boss, and me. Every morning I would stock the kitchen in preparation for the day's menu. This was very good training for me in planning and logistics. There was always a big rush at lunchtime. Larry would bark out the orders to me from the order window, and I would plan how to have all of the items in each order come out simultaneously. After about an hour, the intense rush would be over and then Larry and I could recover and finish out the day. I did the Men's Grill job for two summers before going off to college. I learned many skills from these summer jobs that would serve me well in the future, including planning, logistics, and scale.

Swarthmore College

For college, I went to Swarthmore College, a small liberal arts college on the Main Line suburbs south of Philadelphia, Pennsylvania. Compared to my high school, Swarthmore was tiny—only about 300 students per class year (my high school had 660 students per class). Because of its small size, you knew almost everyone at Swarthmore. In some ways, it was too small

because the familiarity could become stifling. Also, it was a big change from high school—half of the students were valedictorians, and I could no longer coast by. Looking back, Swarthmore was also a very good place for me. Initially, I was interested in both biology and engineering, and so I began as a bioengineering major. After the first year, I realized that I was more interested in biology than engineering, and so I switched my major to biology. Every science course at Swarthmore had a laboratory session attached. From my experience as a faculty member, I now know that this is very rare in universities. In addition, because Swarthmore was small, there wasn't a huge selection of courses for me to take in biology. As a result, I took all of the upper-level core courses at the time: genetics, molecular and cell biology, physiology, invertebrate zoology, and the biology of animal communities (BOAC). Ironically, I really did not enjoy molecular and cell biology or genetics. But this training became important for me many years later. I was especially attracted to the study of animal behavior and ethology because of an influential professor, Kenneth Rawson, who also worked on circadian rhythms.[12] He taught the BOAC course, which was actually an ethology course (the study of natural animal behavior as opposed to psychology, which at the time was dominated by the school of B. F. Skinner and operant conditioning[13]). He had the most amazing laboratory exercises in this course. We did field studies to observe and record the mating behavior of ducks in the spring—repeating the work of Niko Tinbergen (1907–1988),[14] who described fixed action patterns. We studied the work of Konrad Lorenz (1903–1989),[15] who described the phenomenon of imprinting. And, perhaps most compelling, we repeated the experiments of Karl von Frisch (1886–1982),[16] who described the dance language of bees. Dr. Rawson had set up a bee hive encased in plexiglass in the lab with an exit out of the window to the lawn in front of the biology building. We set up feeding stations that had sugar-water feeding stations in different locations in the field. Eventually, foragers bees would come to the feeders and then we would mark the bees using different colored paint spots. Then back at the bee hive, we could observe the forager bees that we had marked at the feeding stations. In the hive, the forager bees would perform their waggle dance, which encoded both the direction of the food source relative to the angle of the sun as well as the distance from the hive. Direction was encoded by the orientation of the waggle dance relative to vertical, which represented the position of the sun. The frequency of the waggles during the waggle dance encoded the distance of the food source from the hive. Amazingly, we could completely replicate these classic experiments done by von Frisch. This experience was eye-opening for me. I had never known the beauty of scientific experiments and the profound insight that could be obtained from studying model organisms such as the honey bee.

The tipping point for me was a lab in the BOAC course on electric fish. As I described, I was an avid tropical fish hobbyist. I had never seen electric

fish before and was fascinated by them. These were weakly electric fish, and there were gymnotids from the Amazon as well as mormyrids from Africa. The lab exercise was to put electrodes into the fish tanks and then visualize the electrical discharges on an oscilloscope. We would then catalog the various electrical discharge patterns of the different species of electric fish. There were about a dozen different species to describe. Some of the fish discharged very high-frequency continuous sine wave signals (*Eigenmania sp.*). Others had low-frequency sporadic discharges (*Gymnotus sp., Hypopomus sp., Elephant nose fish*). I asked Dr. Rawson how long the fish would discharge like this and whether they might rest given that it seemed that continuous electrical discharge might be energy intensive. He said, "why don't we do an experiment and see what they do." So, we set up a chart recorder to the pre-amp and oscilloscope and monitored the frequency of the electrical discharge over time. The first fish that we recorded was a *Hypopomus* electric fish. What we observed was that when left undisturbed, the fish would hide in a tube during the daytime and go completely silent, and then at night it would become active and would have elevated discharge levels. We then put the fish tank in a dark box, and incredibly, a circadian rhythm of electrical discharge was expressed by the fish. I was hooked! How did this happen and did the other species of electric fish also display circadian rhythms?

This experiment occurred during my junior year. Until that point, since I was a biology major, I became a default premed student. My closest friends at Swarthmore, David Mallot and Brooks Martin, were both hardcore premed students and went on to become physicians. So, not really knowing what my options were, I took the premed curriculum. In the fall of my junior year when one would apply to medical school, I learned for the first time that you could go to graduate school in biology. Out of about 40 biology majors in my class, only a couple of us went to graduate school instead of medical school. I told my parents that I had decided not to apply to medical school and instead would pursue research. Of course, my mother was a little disappointed because it was the tradition in the Hara family to become doctors or lawyers, but my parents nevertheless were very supportive of my decision. For my senior year, I did an independent research thesis project on circadian rhythms in electric fish. I went on to measure the electrical discharge patterns of about a half-dozen species of electric fish and wrote a thesis on my findings. At Swarthmore, independent thesis projects were evaluated by an outside faculty examiner at the end of the senior year. My external examiner was Dr. Patricia DeCoursey (1932–2022) from the University of South Carolina.[17] Pat knew Ken Rawson from the University of Wisconsin, where they both had worked with John T. Emlen (1908–1997; father of Stephen T. Emlen at Cornell). Pat was the first person in the field of circadian rhythms to publish a phase response curve to the resetting effects of light pulses in a mammal in 1960.[18] She had left academics to raise a family, but sadly her

husband, George,[19] died prematurely of cancer in his 40s, and Pat had to go back to work. At the University of South Carolina, she was friends with F. John and Winona Vernberg[20] who directed the Belle W. Baruch Institute for Marine Biology and Coastal Research at the University. The Vernbergs arranged a faculty position in the Baruch Institute for Pat, and she then had to find a project relevant to marine biology. Pat needed a research technician and through our interaction at my thesis examination, we connected, and Pat offered me the job. After graduation from Swarthmore, I moved to Columbia, South Carolina, to work with Pat in the Baruch Marine Institute. The year that I spent with Pat was truly wonderful. She became my mentor, and I became her circadian rhythms enthusiast. Although we could not work on circadian rhythms directly, we decided to work on a project to study the daily vertical migration of zooplankton in the water column in the estuary at the Baruch Institute field station. It was a fun project. We built a pontoon boat to conduct the water sampling. Then we designed and built an apparatus to lower into the water and collect water samples at different depths. We then collected samples at different times of day and the tidal cycle. The zooplankton samples were then counted and assessed for vertical migration. When we were back in Columbia on the main campus, Pat and I had countless discussions on circadian rhythms. I also had to apply to graduate school in the fall. At that time, I was interested in three fields: marine biology, orientation and navigation in pigeons, and circadian rhythms. In addition, to working at the Baruch Marine Institute, I had also worked on a National Science Foundation (NSF) summer fellowship at the Chesapeake Biological Laboratory (University of Maryland) in Annapolis, Maryland. The life of a marine biologist was nice: working on the water, being outside, catching fish, and playing volleyball, but I did not find the core work of collecting samples and doing field studies very interesting. It was more observational than experimental. So marine biology ranked third. In the BOAC course taught by Rawson, we studied bird migration and pigeon homing behavior. This led me to visit my Swarthmore friend, John Phillips,[21] who worked in the lab of Kraig Adler at Cornell University in Ithaca and introduced me to the Department of Neurobiology and Behavior, where William T. Keeton (1933–1980)[22] and Stephen Emlen[23] were renown experts studying pigeon homing behavior and celestial navigation in birds. With my love of pigeons as a child in Pakistan, I was very interested in working with Keeton.

For graduate school, I applied to the Scripps Institute of Oceanography at University of California–San Diego (UCSD) where James T. Enright (1932–2004)[24] and Walter Heiligenberg (1938–1994)[25] were faculty; Cornell University in Ithaca where Keeton and Emlen were faculty; Stanford University where Colin S. Pittendrigh (1918–1996),[26] the founder of the field of circadian biology, was in biological sciences; and the University of Texas at Austin (UT Austin) where Michael Menaker (1934–2021)[27] was in zoology. It was an incredibly hard decision for me to choose which of the three

directions to go: oceanography, orientation and navigation, or circadian rhythms. But in the end, I became most interested in circadian rhythms.

University of Texas at Austin and the University of Oregon at Eugene

After applying to work with Michael Menaker, he wrote a letter to Pat DeCoursey saying, "that Takahashi looks pretty good, but can he speak English? … because we would like to support him as a teaching assistant." Pat resolved that question easily. Later that spring, Menaker invited me to Austin for an interview and visit to the lab. I flew to Austin and Menaker picked me up at the airport in his 1954 MG TD roadster. The moment we first met, I knew that Mike was special and that we had much in common. We both went to Swarthmore College, loved nature, and loved sports cars (I had a 1970 Austin Healey Sprite). I knew that Mike was the right person to work with and joined his lab late that summer. Mike's lab was an exciting place in which to work. The lab was engaged in photoperiodism in hamsters led by Jeff Elliot, Milt Stetson, and Fred Turek.[28,29] Till Zimmerman was conducting the pineal transplant experiments that demonstrated the pineal was a circadian pacemaker.[30] The initial work showing the pineal was a circadian oscillator in vitro was being done by Chuck Kasal.[31] Heidi Hamm was working the biochemistry of the melatonin biosynthetic enzyme, N-acetyltransferase.[32] Fred Davis was studying the development of circadian rhythms in mice and hamsters.[33] Faculty who were especially influential for me at UT Austin were George Bittner, Frank Bronson, James Larimer, and George Pollak.

At the time, Menaker was best known for his discovery of extraretinal photoreceptors that mediated entrainment of circadian rhythms in birds.[34] This led to the discovery that the avian pineal gland was essential for the expression of circadian activity rhythms in sparrows,[35] which was the first anatomical locus to be shown to be critical for circadian rhythms in a vertebrate (predating the discovery of a similar role for the mammalian suprachiasmatic nucleus [SCN] in 1972). The lab was also the first to clearly demonstrate that photoperiodic time measurement of reproduction used a circadian clock to measure daylength in a mammal.[28]

I decided initially to explore whether the SCN of birds also played a role in the regulation of circadian rhythms of birds. This would involve surgically lesioning the SCN of birds using house sparrows as the model system because they had robust circadian activity rhythms in constant darkness and because the role of the pineal had been established. At the time, no one in the Menaker lab was experienced in making brain lesions, especially in birds in cases in which the SCN had not been well-described anatomically. In talking to Colin Pittendrigh (Pitt) at Stanford, Mike learned that Pitt had a new postdoc, Rod van Buskirk,[36] who had been a student at the

University of California–Irvine (UC Irvine) in the lab of James McGaugh (see volume 4)[37] and was an expert at making lesions and doing histology. He offered to train me in brain lesioning techniques and histology at Stanford. I was able to spend three weeks with Rod in Pitt's lab learning how to do SCN lesions in mice, perfusing and fixing brains, cutting sections on a freezing microtome, mounting them on slides, and staining them with Nissl. Pittendrigh's lab was also an exciting place. Gene D. Block and Terry L. Page were postdocs in the lab,[38] and Serge Daan (1940–2018)[39] and Pittendrigh had just completed writing the classic five-paper series on the properties of circadian activity rhythms in nocturnal rodents.[40,41] Next door, H. Craig Heller[42] was studying hibernation, and in the same building, was Donald Kennedy's lab,[43] where Gene Block was also a postdoc. Upon my return to the lab, I was able to determine the anatomical location and stereotaxic coordinates of the SCN in house sparrows and to make an extensive set of lesions in the SCN region and study the effects of these lesions on circadian locomotor rhythms.[44] SCN lesions indeed disrupted circadian activity rhythms in sparrows and therefore both the pineal gland and the SCN were essential for the generation of circadian behavioral rhythms in passerine birds.

Mike's lab was the perfect environment for independent and motivated individuals. Mike had many resources that included the main lab on the UT Austin campus in Patterson Laboratories, and he had a "field" lab on the outskirts of town named Balcones Research Center, which was an abandoned magnesium plant built by the federal government during WWII and was acquired and owned by UT Austin. There we had our aviaries for birds and our very large-scale facilities for locomotor activity recording in house sparrows. At the time, Menaker had many hundreds of recording channels with automated computer collection of the data. This technology was led by Ed Kluth, a PhD in physics, who masterminded the data collection system. This was in the late 1970s and computers were only beginning to become available to labs. We used 8-bit programmed data processor (PDP) computers that communicated by punch tape. Despite these ancient relics, we were able to view our computer-generated actograms every week at lab meeting. We then transitioned the activity data collection system from birds to rodents, and many innovations for large-scale recording of circadian activity rhythms were developed by the Menaker lab back then. This system was influential to my career: those of us in the field have spent the past 50 years optimizing and scaling our ability to collect circadian data in an automated fashion ever since.[45]

After I had spent about three and half years in the Menaker lab, Mike was recruited to become the inaugural director of the Institute of Neuroscience at the University of Oregon in Eugene. Mike had been on the faculty at UT Austin since 1962 so you can imagine how much equipment had accumulated and had to be moved and set up in Oregon. Upon making

his decision, Mike hired Sue Williams, a former technician in the lab, to organize and facilitate the move and to set up the new lab in Eugene. The disassembly, packing, and transport of the hundreds of circadian activity chambers was truly a monumental task. The lab equipment filled up two tractor trailer moving vans. In Eugene, Oregon, we also had a "field station" annex to our main lab on campus called the Bio-Social Research Center. The facility was originally built for Dr. John Fentress (1939–2015) to study the behavior of wolves. Slightly off campus, it had a central building that was ideal for housing our circadian chambers, animal colony rooms, and aviaries. Outside were the enclosures designed for wolves, some of which the Menaker lab used for aviaries and others used to house crab-eating macaques from another lab.

After we moved the lab to the University of Oregon at Eugene, Pat DeCoursey spent a sabbatical in the lab, and we conducted action spectrum experiments for phase shifting the clock in hamsters.[46] Although I had attended UT Austin for my first four years of graduate school, I decided to transfer to the University of Oregon and obtained my doctorate in neuroscience in 1981. Oregon was an exciting place. Graham Hoyle, Russell Fernald, Frank Stahl, George Streisinger, Edward Herbert, Chuck Kimmel, Michael Posner, and Monte Westerfield were there. In addition, Jim Simmons, James Weston, Frederick Dahlquist, and Ross Lane were on my doctoral committee in Eugene. For my doctoral thesis, as I've described, I worked on the role of the SCN in the regulation of circadian activity rhythms in house sparrows to test whether the SCN in birds played a role similar to that seen in rodents. In separate work, I also developed a flow-through explant culture system for avian (chick) pineal glands that recently had been shown to express autonomous circadian rhythms of N-acetyltransferase activity.[31,47] To achieve higher time resolution and sampling from individual pineal glands over time series and to determine whether chick pineal glands could express persistent (more than two days) circadian oscillations, we collected culture medium samples continuously for four to seven days with a fraction collector and used melatonin release as the rhythmic output. In Eugene, we were able to expand the flow-through system from 4 channels to 16 channels and expand our melatonin radioimmunoassay capacity to 1,000 samples per week with the able assistance of Sherry Wisner, a research technician in the Menaker lab. At the time, this seemed to be relatively large scale, but we had to use radioimmunoassay of melatonin as our rhythmic output which was tedious and time-consuming. Despite these limitations, we were able to demonstrate that chick pineal glands expressed circadian oscillations of melatonin for at least four cycles in continuous darkness.[48] In addition, the chick pineal gland was responsive to light *in vitro* and this could be seen both as an acute inhibition of melatonin release as well as the enhancement of melatonin rhythms when maintained in LD12:12 light-dark cycles in culture. Thus, the chick pineal contained an entire circadian system:

(1) a photic input pathway; (2) a circadian oscillator; and (3) an output pathway for melatonin synthesis.

I completed and defended my doctoral thesis in 1981, and I believe that I was the first doctoral student to graduate from the Institute of Neuroscience at the University of Oregon, Eugene, which began in 1979.

Mike was a big thinker and was always interested in evolution and "why" things in life were the way they were. He gave us great latitude in the lab and was not detail oriented. Indeed, we all knew that Mike was not a hands-on mentor and was happy to admit that he was not very good with his hands. Instead, Mike trained our minds. He was a critical thinker and our lab discussions about every subject were sharply focused on logic and evolutionary biology, which of course is not always logical. Many great ideas were discussed at length and then each of us would try to design and organize the best experiments to test our ideas. If you could make a good case for an experiment, then Mike would give you the green light and you were off to the races. It was a wonderful environment to conduct research and to learn the ways of the scientific world. Mike let us all in on the inner workings of the faculty and department politics. Thus, Mike trained us not only in how to do good science but also in how to survive and maneuver the world of publishing, grant writing, and university politics. Finally, Mike was an exceptional writer, and he took great pride in writing and tried to transfer these skills to us. In retrospect, I can see clearly how rare this was and how fortunate we as trainees of Mike have been.

Sadly, for the field of circadian biology, Michael Menaker passed away on February 14, 2021.[27] One profound regret that I have (despite continuous nominations since 2004) is that Mike was not elected into the National Academy of Sciences, which was a distinction that he clearly deserved. Since 2003, more than a dozen individuals working in the field of circadian rhythms were elected to the NAS, including Jeff Hall (2003), Michael Rosbash (2003), Joe Takahashi (2003), Woody Hastings (2003), Anthony Cashmore (2003), Aziz Sancar (2005), Mike Young (2007), Steve Kay (2008), Jay Dunlap (2009), Susan Golden (2010), Louis Ptacek (2012), Xinnian Dong (2012), Amita Sehgal (2016), Mitch Lazar (2017), Ying-Hui Fu (2018) and Ueli Schibler (2022). The most obvious and prominent omission from the NAS in our field is Michael Menaker.

NIH and NIMH

During my final year at the University of Oregon, I was awarded a Pharmacology Research Associate Training Program (PRAT) fellowship for two years from the National Institute of General Medical Sciences at the NIH. I decided to work with Martin (Marty) Zatz who was a member of Julius (Julie) Axelrod's (see volume 1)[49] Section on Pharmacology in the Laboratory of Clinical Science in the National Institute of Mental Health

(NIMH). Marty worked on the beta-adrenergic regulation of adenylate cyclase and N-acetyltransferase in the rat pineal gland with Julie,[50] and we decided to apply his expertise on adrenergic receptors and cyclic nucleotides to the chick pineal circadian oscillator. I was Marty's first postdoc, and the lab was made up of the two of us. The lab was a single room with a lab bench and two desks at each end at which Marty and I sat. This was embedded within Julie's Section on Pharmacology, which was in turn embedded within the much larger Laboratory of Clinical Science led by Irwin Kopin.[51] We focused on photic and circadian regulation of cyclic nucleotides in the chick pineal gland *in vitro*.[52] In addition, I collaborated with Arnold Eskin at the University of Houston on the serotonergic and cyclic nucleotide regulation of circadian rhythms in the *Aplysia* eye, which at the time was the best *in vitro* preparation for studying circadian oscillations.[53,54]

Marty was a character to say the least. We initially got along very well, but after a while, things became more tense. I was used to having a great deal of independence in the Menaker lab, which was a large freewheeling intellectual and physical space. The contrast with Marty's lab was stark. Unlike Mike Menaker, Marty was very hands on and was involved in everything that I did. After about a year or so, this became stifling for me and led to some disagreements that distanced us until we resolved them many years later.

During my second year at NIMH, Julie's section became part of Michael Brownstein's Laboratory of Cell Biology, and Marty and I became members of the Brownstein lab. There I learned critical skills from Tomas Hökfelt,[55] a renowned scientist visiting from the Karolinska Institute, and Miklós Palkovits, the famous Hungarian anatomist. From Hökfelt's technician, Gun Norell, I learned immunohistochemistry, and from Palkovits,[56] I learned rapid freehand dissections of rodent brains that would later be important for me to apply to the SCN.

Because I had a two-year fellowship at NIH, and I did not want to continue working there, I was exploring postdoctoral opportunities with Denis Baylor at Stanford. From my work with Pat DeCoursey and Menaker at Oregon where we measured the first action spectrum for phase shifting the circadian clock in a mammal,[46] I had become extremely interested in phototransduction mechanisms. King-Wai Yau and Denis Baylor (1940–2022) had just published classic papers on the biophysics of phototransduction in isolated rod photoreceptors.[57,58] I vividly remember visiting Baylor's lab at Stanford and was very fortunate to meet Lubert Stryer who had recently discovered the mechanism of photoactivation of the G-protein transducin.[59] Stanford was an amazing place. In addition, my sister, Nancy Takahashi, was an undergraduate at Stanford from 1977 to 1981, where she studied architecture and urban design and was on the Council of Presidents from 1980 to 1981. I visited Nancy on campus many times and came to love the Stanford campus.

Northwestern University

At the same time, however, Fred Turek at Northwestern University recruited me to apply for a faculty position in the Department of Neurobiology and Physiology on the Evanston campus. Fred was a former postdoc with Menaker, and he and I overlapped in the Menaker lab for about a week. I remember going to Fred's going-away celebration at Scholz Beer Garden in Austin, Texas. Fred likes to say that we were a tag team. He took care of Menaker's lab when he was a postdoc and then handed it over to me when I began in 1975. I applied for the job at Northwestern and was interviewed and then was offered a tenure-track position as an assistant professor. This event changed my original plan to go to Stanford, and I accepted the position at Northwestern and began in July 1983. My lab was next door to Fred's lab in an "interesting" building known as Hogan Hall. The architect's concept of the building for scientists was a monastery. He designed the building in a manner that reduced interactions because he believed that scientists would want to work in isolation without distractions. What could be further from the truth for those of us in science? In any case, Fred's lab and mine shared a hallway entrance so thankfully we were not isolated from each other. Ironically, our two labs occupied the space that had previously been Frank Brown Jr.'s laboratory. This was ironic because Frank Brown did not believe in endogenous circadian oscillators and hypothesized that the 24-hour daily rhythms in living systems were driven in response to subtle geophysical cues from the earth's rotation.[60] He and his disciples argued strongly against the existence of endogenous circadian clocks and believed all 24-hour rhythms were exogenously controlled. Although Brown's hypothesis was the antithesis of the work from the founders of our field, Colin Pittendrigh and Jürgen Aschoff (1913–1998),[61] Frank Brown was a true gentleman on the few occasions that I was able to meet him. After Frank Brown's retirement, the exogenous hypothesis of circadian rhythms gradually faded away and today is buried under a mountain of evidence that circadian clocks are endogenous and are genetically determined.

When I started my new lab at Northwestern, I was interested in two areas of focus: (1) the sensitivity and integration of photic stimuli that entrain circadian rhythms in rodents; and (2) the cellular and molecular basis of circadian oscillations using the chick pineal as a model system. To study the photic entrainment of rodents, we used golden hamsters because of their extremely precise circadian rhythms of wheel-running activity. My first graduate student, Dwight Nelson, took on this project, and we focused on the threshold and integration of light stimuli that induced phase shifts in the circadian rhythm in constant darkness. Dwight performed beautifully quantitative experiments on the integration of intensity and duration for light pulses that reset the hamster clock.[62] He showed clearly that hamsters could integrate light information for tens of minutes and that the

optimal integration time was 300 seconds. Pulses either shorter or longer in duration were less effective in phase shifting and required more photons to achieve comparable phase shifts to that seen with 300-second pulses. The integration time of this photoreceptive system was 100 times longer than the known integration time of rod (1 second) or cone (100 milliseconds) photoreceptors, raising the question "at what stage in the photic pathway is the long integration time achieved?" The long integration time, high threshold, and spectral sensitivity curve for phase shifting was not consistent with the known photoreceptors in the hamster at the time. Indeed, in retinal degenerate mice, Russell Foster and colleagues showed that circadian phase-shifting responses to light were intact in the absence of rods and most cones,[63] strongly indicating that there must be additional photoreceptors in the retina. In 1998–2000, Ignacio Provencio discovered the novel photopigment, melanopsin, which was expressed in a subset of retinal ganglion cells in the retina.[64] In 2022, David Berson and Samer Hattar provided compelling evidence that melanopsin-containing retinal ganglion cells were intrinsically photosensitive,[65,66] and subsequent work from many labs has firmly established that the mammalian retina has three classes of photoreceptors: rods, cones, and intrinsically photosensitive retinal ganglion cells (ipRGCs).[67,68] Interestingly, the long integration time for the photoresponse for phase shifting can be seen in the spike output of ipRGCs and the Ca^{2+} responses of SCN neurons (Michael Tri Do, pers. comm.).

To study the cellular and molecular basis of circadian clocks in vertebrates, I chose to use the chick pineal gland as a model system because it contains all three elements of a circadian system (an input pathway, a circadian oscillator, and an output pathway) that could be studied *in vitro*. Upon setting up my lab, we focused on using primary dissociated cell cultures from the chick pineal. Cultured pineal cells were much more robust than pineal explants and allowed us to scale up these experiments using 96-well plates. Barbara Pratt, a talented postdoc, was able to study alpha-2 adrenergic and vasoactive intestinal polypeptide (VIP) regulation of melatonin and cyclic AMP in pineal cells.[69,70] My graduate students, Linda Robertson and Selene Nikaido, were able to use perfused pineal cells grown on microcarrier beads to study circadian photic entrainment by light and circadian regulation of cyclic AMP synthesis and release.[71-73] We could study many aspects of the circadian clock in pineal cells, including the role of macromolecular synthesis and cyclic nucleotide and Ca^{2+} regulation.[74] Unlike the *Aplysia* eye circadian clock,[53] however, cyclic nucleotides did not reset the oscillator as an input but instead regulated melatonin synthesis in the output pathway. Protein synthesis inhibitors, however, were potent resetting agents and their phase-shifting action mimicked the effects of dark pulses (the opposite of light pulses).[74] We explored the role of protein synthesis in both rodents and chick pineal cells and found that protein synthesis inhibitors also mimicked dark pulses in hamsters.[75] The locus of action was the SCN,

as demonstrated by microinfusion of anisomycin into the SCN region.[76] In 1990, the labs of Benjamin Rusak and William Schwartz reported that light exposure induced c-FOS expression in the SCN.[77,78] Jon Kornhauser, a graduate student who worked with Kelly Mayo and me, rapidly followed up on the c-FOS experiments, and we showed that *c-fos* induction in the SCN was quantitatively correlated with the magnitude of phase shift induced by light in hamsters.[79] We also found that the induction of *c-fos* was dependent on the circadian phase and was only induced during the subjective night. Jon went on to show that *jun-B* was co-induced with *c-fos* in the SCN and that AP-1 DNA-binding activity was light induced in the SCN.[80] Soon after, we were contacted by Mike Greenberg and David Ginty about using the light response in the SCN to study Ser^{135} phosphorylation of CREB using a phosphospecific antibody that David had recently created.[81] The three of us (David, Jon, and I) performed the experiments together and found a rapid phosphorylation of CREB in cells of the SCN following light exposure of animals to light. Like the *c-fos* and AP-1 response, CREB phosphorylation was also "gated" by the circadian clock in the SCN. This work led to a long-term friendship with both David Ginty and Mike Greenberg.

In addition, to these two lines of inquiry, I also delved into identifying melatonin receptors. Margarita Dubocovich and I developed a high-affinity binding assay for melatonin receptors using 2-iodomelatonin as a ligand. I had used iodinated melatonin analogs for radioimmunoassays for our chick pineal melatonin flow-through experiments, and we had to radioiodinate the melatonin analog for the radioimmunoassay ourselves. Thus, it was easy for me to radioiodinate 2-iodomelatonin as a ligand for receptor binding assays. I synthesized and purified the 2-[^{127}I]iodomelatonin ligand, and Margarita did the binding assays. It worked like a charm. We were able to define high-affinity melatonin receptors using this ligand and published a two-author paper in 1987.[82] Subsequently, this created a cottage industry for melatonin receptors and the ligand could be used to identify melatonin receptors in the brain using autoradiography.[83]

During this time, I met my first wife, Barbara Snook, who was a nurse at Evanston Hospital and who was going back to finish her bachelor's degree at Northwestern. Barbara graduated from Northwestern and then went to medical school at Tufts University in Boston. Barbara was from Northampton, Massachusetts, and came from a line of physicians. Her grandmother was a medical doctor and was the first female medical examiner in the state of Massachusetts. Her father, George A. Snook (1925–2017),[84] was an accomplished orthopedic surgeon and team physician at the University of Massachusetts in Amherst. After one year at Tufts, we decided to get married in 1985 and Barbara transferred to Northwestern Medical School and graduated in 1988. We lived in a university house very near to campus in Evanston. Barbara decided to do a residency in pathology at Evanston Hospital. She was interested in clinical pathology, but Evanston was stronger

in surgical pathology, so the first year was pretty brutal with constant autopsies to be performed. Once Barbara became pregnant with our first child, Erika, who was born in 1991, she decided to take time away from medicine and devoted herself to raising Erika. In 1994, our son, Matthew was born, and Barbara then became a full-time mother to our two children. At that time, we finally were able to buy our own house and we moved to Wilmette, Illinois, into a very nice neighborhood on the North Shore. Once the kids were in school, Barbara then pursued one of her interests and went to the University of Illinois Chicago to get a master's degree in public health. Erika and Matthew continued to be the center of her attention; therefore, Barbara did not pursue her career in medicine. She used her expertise in medicine and public health to volunteer for Congresswoman Jan Schakowsky (Ninth District, Illinois).

Once the children had grown enough, I introduced them to my two athletic passions, tennis and skiing. I was in my 40s at this time and having to focus intensely on the lab meant that staying in shape went by the wayside. After playing tennis, I noticed that my knees were sore, and the first signs of age were creeping in. This was an important wake-up call for me because I realized that I needed to pay more attention to my health and fitness. So along with tennis, my tennis pro, Jill Bachochin, who was very intense, became my personal trainer. She completely whipped me into shape, and magically, my knee pain went away. After Jill transferred to another tennis club, I had the good fortune to have Michelle Casati as my tennis pro. Michelle was on the pro tour for 10 years (ranked as high as 18th in the world) and was the best coach I have ever had. Since that time, I have always stayed in shape, and 30 years later my knees are still fine. Skiing has been more of a challenge both in having opportunities to be able to ski and remembering that I am no longer that teenager who could blast down the black diamond slopes with moguls willy-nilly. Now the blue runs are more my speed.

Search for Circadian Proteins and the Turning Point

Returning to circadian biology, based on the resetting effects of protein synthesis inhibitors in every circadian system that had been studied, the photic induction of immediate-early genes in the SCN, and the discovery of the *period* gene in *Drosophila*,[85] I was convinced that we had to find "circadian" genes and proteins. An extremely talented MD/PhD student, Jose C. Florez, joined my lab, and Jose decided to use two-dimensional (2D) gel electrophoresis to find oscillating proteins in chick pineal cells. We adopted the quantitative 2D-gel electrophoresis method developed by Jim Garrels at Cold Spring Harbor Laboratory,[86] and obtained a system from Protein Database Inc., which at the time was the state-of-the-art system. Jose conducted beautiful 2D-gel experiments and was able to quantitate

700 radiolabeled proteins from chick pineal cells.[87] A number of proteins changed significantly during the circadian cycle, but two (p56 and p22) in particular were most striking. Jose was able to use preparative 2D gels and isolate the p56 protein for peptide sequencing. The identity of p56 was tryptophan hydroxylase, which was confirmed by immunoblotting. Although we had achieved our goal of finding newly synthesized circadian cycling proteins, the result was disappointing because tryptophan hydroxylase is on the output pathway for the synthesis of melatonin. We did not know previously that it oscillated, but unfortunately, it was not a component of the circadian oscillator mechanism. Parenthetically, Jose Florez went on to have an incredible career on the genetics of type 2 diabetes and is now the chair of Medicine at Massachusetts General Hospital.

The 2D-gel experiments of Jose Florez were a turning point for me. Here we had used the most sophisticated biochemical method for detecting circadian rhythms in newly synthesized proteins. Jose could analyze more than 700 proteins, yet we could identify only a handful of them by peptide sequencing. Furthermore, a cell expresses more than 10,000 proteins and we could "see" only the top 700. That left the majority of the proteome invisible to us. There had to be another way to find circadian clock proteins. I came to realize that the only way that you could find a gene or protein without any preexisting information is genetics. You need a clear phenotype that you understand, but you do not need any mechanistic understanding of the phenotype as long as it is genetically transmitted (heritable). From following the discovery of the *period* locus in *Drosophila* by Ron Konopka and Seymour Benzer,[88] it was clear that single genes could control circadian rhythmicity. In addition, the discovery of a spontaneous mutant in the golden hamster that had a 20-hour period length clock demonstrated that, like *Drosophila*, rodents could also carry single-gene mutations with strong circadian clock phenotypes.[89]

Genetics, My Second Career

Lawrence (Larry) H. Pinto in my department at Northwestern was using mouse mutants to study the visual system. Larry knew William (Bill) F. Dove and Alexandra (Alex) Shedlovsky (1933–2023) in the McCardle Center at the University of Wisconsin, Madison, who were conducting *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screens for an enzyme, phenylalanine hydroxylase. Larry, Fred Turek, and I decided to meet with Bill Dove and Alex, and we set up a collaboration to perform an ENU screen for circadian activity rhythms in mice. J. David McDonald in Bill and Alex's lab performed the ENU mutagenesis and conducted a dominant screen for phenylalanine hydroxylase mutants using a C57BL/6J genetic background. After screening the mice in Madison, they shipped the mice to Evanston, and Martha Vitaterna, a talented postdoc in my lab, screened the mice for

circadian phenotypes using our standard wheel-running activity assay. At the time in about 1990–1991, I decided to "invest" up to 100 wheel-running cages to this screen. This was a modest investment because we had many hundreds of wheel-running cages at our disposal. Our thinking at the time was that the ENU mutation rate was about 1 in 700 per locus per gamete and that if we screened 2,100 mice, we might achieve 3× coverage of the genome at this mutation rate. With 100 cages and a four-week phenotyping time for each mouse, we could screen 1,200 mice per year and achieve our goal of screening 2,100 mice in less than two years.

Despite the work of Konopka and Benzer (1971)[85] showing that mutations in a single locus could either abolish, shorten, or lengthen circadian rhythms in *Drosophila*, there was great skepticism that ENU mutagenesis could be used to isolate behavioral mutants in mice. ENU mutagenesis had been used only to identify lethal and overt mutations in mice. There were a few prevailing notions that led to this skepticism. First, behavior was thought to be a complex trait that was polygenic and unlikely to be driven by single genes. Second, most behavioral assays in rodents were devised for ease and convenience in the laboratory rather than for more ethological or naturalistic considerations. Thus, many behavioral tests for locomotion, anxiety, and learning and memory employ tasks and arenas that are contrived and artificial. As I have written elsewhere,[90] the variance in quantitative assessments of many behavioral assays is significant and therefore many behaviors are too noisy for screens. For example, a gold standard assay for learning and memory is context-dependent fear conditioning. In later work,[91] screening many thousands of mice for defects in context-dependent fear conditioning, we found that the standard deviation for the percent freezing score was about 20 percent, with a floor of 0 percent and a ceiling of 100 percent freezing. With a mean level of 50 percent freezing, the greatest deviation possible would be 2.5 standard deviations from the mean or a *Z*-score of 2.5, equivalent to a coefficient of variation of 40 percent. Despite these general concerns, we were fortified with the knowledge that our circadian phenotype was orders of magnitude less variable (0.17 hour standard deviation divided by a mean of 23.7 hour = 0.00717 or a coefficient of variation of 0.7 percent). In addition, as I have discussed elsewhere,[92] the key circadian phenotype is period length. This phenotypic measure is much less prone to artifacts because the overt periodicity that we measure at the behavioral level is determined by the periodicity (timing) of the underlying oscillator, even though the output pathway may be complex or many steps away from the oscillator. Other circadian parameters, such as the level or amplitude, can be affected independently of the oscillator and thus are less reliable phenotypes. Finally, the coefficient of variation of phenotypes, such as level or amplitude, are much larger than that seen for circadian period.[90]

Armed with this knowledge, we undertook the circadian ENU screen, and Martha tested about 40 mice in the first batch. Mouse #25 in this

batch expressed a circadian period of 24.8 hours in constant darkness. This was 1 hour longer than wild-type (WT) mice and was 6 standard deviations away from the mean circadian period of the screening population (23.7±0.17 standard deviation).[93] Because we found this mouse very early in the screen, we only screened 304 mutagenized mice. Test crossing this male mouse by conducting a backcross to WT females revealed that about half of the offspring carried the 1-hour-long phenotype confirming that the mutant phenotype was heritable and consistent with a dominant mutation. Intercrossing two presumptive heterozygous carrier mice then produced litters that might carry homozygous mutations. Indeed, in the very first F2 litter, there were four mice born, and upon circadian phenotyping, there were (phenotypically) one WT, two heterozygotes, and one homozygous mutant—a perfect 1:2:1 ratio for a semidominant Mendelian trait. This was the first homozygous mutant that we observed, and its phenotype was spectacular. The mouse could entrain almost normally on LD12:12, but upon release into constant darkness, it had a 28-hour circadian period, waking up 4 hours later each day. After about two weeks in constant darkness, the 28-hour circadian rhythm damped out and became arrhythmic. Thus, the mutation was semidominant for circadian period length with heterozygotes having a 1-hour-longer period and homozygotes having a 4-hour-longer period length. Light pulses could transiently restore the rhythm in constant darkness and reexposure to LD12:12 could restore a diurnal rhythm. We went on to map the mutant genetically to mouse chromosome 5 using the simple sequence length polymorphism (SSLP) map that had just been created in Eric Lander's mouse genome center.[94] We named this mutant "*Clock*," which ultimately was an acronym because one of the reviewers of our paper said it was too early to know whether the mutant was directly related to the circadian clock. The name of the gene then became "*Circadian locomotor output cycles kaput*" or *Clock* based on the phenotype of the mutant, which we published in 1994.[93]

Discovery of the Clock *Gene*

Once we had isolated the *Clock* mutant mouse, which had a long circadian period phenotype that was comparable to the longest period mutants seen in *Drosophila*, we knew we were on to something. I began to change the focus of the lab to mouse genetics. We had already made the transition to molecular biology in the early 1990s, and now with the *Clock* mutant in hand, we could use this mutant to find the underlying gene using positional cloning. In the mid-1990s, positional cloning of mouse and human genes was nontrivial.[95] High-resolution genetic maps were only completed for the mouse in 1996,[96] physical maps of the mouse genome in 1999,[97] and the genome sequence of the mouse was not completed until 2002.[98]

The first step in positional cloning was to map the *Clock* mutation with high enough genetic resolution (less than 1 centimorgan) to identify the genomic region of the mutant locus so that the genomic DNA could be cloned physically. David King, a talented graduate student in the lab, led this effort with Martha Vitaterna who made mapping crosses to two other inbred strains of mice to allow genotyping of the crosses. Bill Dietrich in Eric Lander's lab was very generous in sharing his SSLP genetic mapping information and advising us along the way as he was creating the high-resolution genetic map of the mouse genome.[96] In the end, we collected about 2,600 meiotic events to fine map the *Clock* mutant to a 0.3-centimorgan interval in the middle of mouse chromosome 5. During this mapping process, it was clear that the task of positional cloning would require a team effort. There were too many different aspects of the project to do and too many different skill sets required for success. Thus, about a dozen members of my lab worked together as a team over a three-year period (1994–1997) to perform all the various steps required to find and clone the *Clock* gene (fine mapping, physical mapping, transcription unit identification, gene structure, cDNA identification, identification of the causative mutation, and transgenic mouse methods to confirm the identity of candidate genes as causal). I described the cloning of the *Clock* gene to the lab as a restriction point that we had to go through. On the other side, everything would open up, and each of the lab members could run with the fruits of the discovery.

I was fortunate to be introduced to Jeff Friedman at Rockefeller by Michael Young during a seminar visit there. Jeff had just cloned the mouse *obese* (*ob*) mutant using positional cloning and was in the process of publishing their landmark paper in *Nature*.[99] We were completing our high-resolution genetic mapping of *Clock* and transitioning to physical mapping of the region. Once we had fine mapped *Clock* to the midpoint of chromosome 5, we found that no one had been there before, and the region had not been cloned or physically mapped. In addition, very few of the genes in the region had been annotated. A large deletion mutant (W^{19H}) in the region contained the *Kit* locus that was 0.7 cM proximal to *Clock*.[100] Complementation crosses of *Clock* with W^{19H} showed that *Clock* was located within the W^{19H} deletion; however, we estimated that W^{19H} was about 2.8 cM in size, which did not narrow the critical region containing *Clock* sufficiently. Importantly, the deletion mapping of *Clock* allowed us to determine that the *Clock* mutant allele behaved as an antimorph.[100] The W^{19H} deletion allowed us to observe the effect of a single copy of the *Clock* mutant allele in the absence of a WT allele. This *Clock*/deletion phenotype was more severe than the phenotype of *Clock*/+ heterozygotes, but it was less severe than the phenotype of *Clock*/*Clock* homozygotes. In addition, the +/deletion hemizygous phenotype was indistinguishable from the homozygous WT phenotype. Thus, the null allele was recessive to the WT allele. The relative severity and order of

these mutant phenotypes is the defining characteristic of an antimorphic allele.[101] This knowledge would be important for us later when we needed to rescue the *Clock* mutant to prove its gene identity.

Armed with this high-resolution genetic map, the next step was to map the genomic region physically. Yeast artificial chromosome (YAC) libraries of the mouse genome were available, and we were able to isolate a 1-megabase-long YAC clone that spanned the genetic interval containing *Clock* (assuming that we had not made any mistakes in genotyping recombinants that closely flanked the *Clock* locus). In talking to Jeff Friedman, he had just used bacterial artificial chromosome (BAC) clones to physically map the mouse *ob* region and encouraged me to use BACs to physically map our region. Although we did have a YAC clone spanning the region, YACs were very difficult to purify and manipulate (requiring pulse-field gel electrophoresis) and were unstable. In addition, many YAC clones were known to be chimeric (containing inserts from different parts of the genome) and therefore might be unreliable. We were able to screen a mouse BAC library using our genetic SSLP markers as sequence tagged sites (STS) and created a BAC contig physical map of most of the nonrecombinant genetic interval containing *Clock*. BAC clones were very stable and bacterial expression facilitated the recovery of significant amounts of DNA.

To find transcription units (genes within the region), two methods were used at the time: exon-trapping and cDNA selection. We decided to focus on cDNA selection because Jon Kornhauser and I had made cDNA libraries from the mouse SCN in order to find genes that differed between dark and light exposure of mice. We assumed that circadian clock genes should be highly expressed in the SCN because this was the anatomical locus of the circadian clock in mammals.[102] Yaliang Zhao, a graduate student in the lab, performed the cDNA selection experiments. She used BAC clones as probes to screen the SCN libraries directly. In a second method, we developed a cDNA selection method based on the DNA subtraction method, representational difference analysis (RDA), to isolate cDNA fragments that hybridized with BAC DNA from the contig. Phil Lowrey in my lab was using genetically directed RDA to clone the hamster *tau* mutation, which we achieved a few years later in 2000.[103] In the end, cDNA selection worked and was extremely valuable in reconstructing the genomic structure of the *Clock* gene; however, it was not a comprehensive method and required the gene to be expressed in order to identify it. Therefore, we undertook a third method, which was to shotgun sequence BAC clones in the critical region. Lisa Wilsbacher and Ashvin Sangoram, two talented MD/PhD students, took on this aspect of the project. They purified BAC clones from the contig and made M13 shotgun libraries for single-stranded sequencing. This method turned out to be the best and most robust method for identifying genes in the contig. In addition, we used this genomic sequence to determine the gene structure of

all the genes in the contig. A few years later, Lisa published the complete sequence and comparative analysis (mouse and human) of a 204 Kb interval containing the *Clock* locus.[104] Ironically, when we deposited this genomic sequence in GenBank, we were in the top 10 of groups depositing mouse genomic sequence—the rest of the list were all genome centers.

As we steadily created our genetic and physical map of the genomic region containing the *Clock* gene, we knew that we would need independent evidence that a specific candidate gene was the causative gene. For positional cloning in human genetics, independent evidence was satisfied by identifying two independent causative (with a phenotype) mutations in the same gene. In our case, we only had one mutation; therefore, we would need another way to confirm that the gene was causative. The best approach was to try to rescue the mutant using a WT version of the candidate gene. I had learned in conversations with Eddy Ruben at Lawrence Berkeley Labs and Nat Heintz at Rockefeller that one could make BAC transgenic mice reliably that contained very large inserts of 100–200 Kb. Doug Engel at Northwestern had also successfully made transgenic mice with YAC clones that were about 300 Kb in size. I was fortunate that I had two extremely talented research technicians, Eun-Joo Song and Weimin Song (later), who could make transgenic mice by pronuclear injection, and my lab had already set up a microinjection facility because our core facility was not efficient in creating transgenic or ES-cell derived mice at that time. We selected BAC clones that covered a 200 Kb critical region that should contain the *Clock* gene to make BAC transgenic mice using *in vivo* complementation as a functional assay for the *Clock* gene. Because previous work had shown that the mutant *Clock* allele was antimorphic (a "competitive" type of dominant-negative mutation),[100] we reasoned that it should be possible to rescue the *Clock* mutant phenotype by overexpression of the WT gene. Initially, we made three different BAC transgenic mouse lines. One of these lines containing a 140 Kb BAC transgene (BAC 54) completely rescued the *Clock* mutant phenotype. I remember the lab meeting in August 1996 vividly. Marina Antoch presented the results of crossing the BAC transgenic mice to *Clock* mutant mice. Of the three transgenic lines, only BAC 54 rescued the *Clock* mutant phenotype. This was a watershed moment because the rescue experiment showed beyond a shadow of doubt that the *Clock* gene was located within a 140 Kb interval. Until this point in time, we had hoped that our genetic and physical mapping was correct, but there was always the possibility that one or more recombinant mice had been mistyped and our genetic map of the critical region could be off. The rescue of the *Clock* mutant phenotype with a 140 Kb physical piece of DNA proved the gene was there. Subsequent shotgun sequencing of BAC 54 by Lisa Wilsbacher and Ash Sangoram revealed that there was only one gene located in the BAC. The gene turned out to be large, covering about 100 Kb genomically.

Together the team, using cDNAs from the region and the genomic sequence of BAC 54, found that the *Clock* gene contained 24 exons (I joked later: one exon for every hour of the day) and encoded a basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) domain protein. The predicted amino acid sequence of the *Clock* gene strongly suggested that it was a transcriptional activator. Looking back, this was clearly the highlight of my career. Despite the naysayers, we had conducted a forward genetics phenotypic screen for a circadian mutant mouse to isolate the *Clock* mutant mouse, and we had used this mouse to find the causative gene using positional cloning and *in vivo* complementation using transgenic BAC mice.

We prepared two manuscripts of this work: one on the positional cloning and a second on the *in vivo* complementation of the *Clock* mutant. We submitted the manuscripts to *Science* magazine, where Katrina Kelner was the editor handling our submission. Shortly afterward, I made our first public announcement of the cloning of the *Clock* gene at a Neuroscience Symposium at the NIH organized by Zach Hall in March 1997. I spoke in a session in which I was preceded by Eric Kandel. I presented our work, and the response was overwhelmingly positive. Interestingly, within 24 hours of my talk, Ben Lewin of Cell Press contacted me stating that he had heard that we had cloned the *Clock* gene and that he would be very interested in having us submit our work to *Cell*. Of course, this was a dilemma because we had already submitted an editorial inquiry to Katrina Kelner at *Science*. I called a lab meeting, and we discussed this exciting invitation to submit our papers to *Cell*. The team unanimously thought that publishing in *Cell* would be preferable to *Science*. Therefore, I withdrew our submission to *Science,* and we submitted to *Cell*. Lewin reviewed our manuscripts in less than two weeks and accepted them pending minor revisions and offered us the cover. Thus, on May 16, 1997, our two papers were published in *Cell*.[105,106] In retrospect this was a very good decision because our two papers spanned 26 pages in *Cell*. Had we published in *Science*, I am fairly certain that they would not have given us 26 pages to publish our papers.

As I mentioned before, there was always great skepticism that our approach to finding circadian clock genes in mice would work. First, there was the bias that single genes would not strongly affect circadian behavior because it was "complex." Second, once we had isolated the *Clock* mutant mouse, there were two forms of skepticism: (1) we would not be able to clone the gene because positional cloning was too difficult (and we had better ask Eric Lander to help us clone the gene!); and (2) even if we succeeded in cloning the gene, it would not be very informative—it might be Calmodulin Kinase II. Well, we proved the skeptics wrong. The moment our paper appeared in *Cell*, the entire attitude to our work changed. Everyone now applauded it, and the skepticism and naysayers disappeared. This was truly one of the most enjoyable times of my career.

Howard Hughes Medical Institute

May of 1997 was also a signal moment for me because I was chosen to be a Howard Hughes Medical Institute (HHMI) Investigator with my appointment beginning on July 1, 1997. I considered it a high privilege to be able to work within such an august organization. My appointment to HHMI was not my first interaction with the institute. Donald Harter who was the HHMI scientific officer for Northwestern University was surprised that I had not been nominated in the 1994 HHMI competition. At that time, HHMI had just switched from institutional appointments to competitions for HHMI appointments. The competitions were limited to a set of universities, however, and each entity was allowed to nominate a finite number of candidates. In the case of Northwestern, only two candidates could be nominated and, in 1994, I was in line for nomination but two individuals were ahead of me. Meanwhile in 1996, I was recruited to Duke University and Baylor College of Medicine. There were four remaining institutional HHMI appointments in the country in 1996, and I was offered HHMI positions at both Duke and Baylor. My first wife, Barbara, was a New Englander and was not inclined to move to the south, so I eventually declined both positions, which was incredibly difficult for me because I was turning down guaranteed positions as an HHMI Investigator. In retrospect, these decisions were likely beneficial because in 1996 we had not finished cloning the *Clock* gene and moving the lab at that time would have been very disruptive to our effort to clone the gene. Luckily for me, I became an HHMI investigator in the 1997 competition.

NSF STC Center for Biological Timing

From 1991 to 2002, I was privileged to be a member of an NSF Science and Technology Center (STC) led by Gene Block at the University of Virginia (UVa) called the Center for Biological Timing. There were three institutions in the Center: UVa, Northwestern, and Rockefeller with Mike Young. This NSF STC was critical for the field of circadian biology because it allowed for a new effort that I led called the "Clock Genome Project." This project involved conducting forward genetic screens for circadian mutants using *Arabidopsis*, *Drosophila*, and mouse. Steve Kay had created a circadian promoter::luciferase reporter in *Arabidopsis,* and Andrew Millar and Steve had isolated all of the critical circadian mutants in *Arabidopsis* during this time. Mike Young had been conducting screens for circadian mutants on the *Drosophila* autosomes, and eventually, we recruited Michael Rosbash and Jeff Hall at Brandeis into the NSF STC to corner the market in *Drosophila* circadian labs. The NST STC funded our work on cloning the *Clock* gene as well as the cloning of the hamster *tau* gene (which was a missense mutation in Casein Kinase 1 epsilon). At that time, it was not possible to fund

this work through the NIH because it was too risky. Indeed, David King, Phil Lowrey, and Sharon Low all had their F31 NRSA applications declined because they were "too ambitious and too risky." David was the first author of the 1997 *Cell* paper positionally cloning *Clock*; Phil was the first author of a *Science* article cloning the hamster *tau* mutant using genetically directed representational difference analysis; and Sharon was the first author of a two-author paper in *Cell* using chimeric mice to study the interaction of wildtype and *Clock* mutant cells *in vivo* for the generation of circadian activity rhythms. Sharon was awarded the Donald B. Lindsley Prize in Behavioral Neuroscience in 2002 from the Society for Neuroscience which is awarded to the best doctoral thesis in neuroscience in the previous year. These examples, exemplify the conservative nature of the NIH review process. Thank goodness we did not depend on the NIH to support our work on these "ambitious and risky" endeavors!

Mechanism of the Circadian Clock in Mammals

Going back to 1997, I contacted Mike Young, Michael Rosbash, and Jeff Hall and informed them that we had cloned the *Clock* gene and asked them if they would like to collaborate to identify the *Drosophila* ortholog. All three said they were too busy and that they would have to remove a student or postdoc from a project to take this on. So, Steve Kay and I decided to find the fly orthologs ourselves. We rapidly found fly-expressed sequence tags (ESTs) similar to mouse *Clock* and were able to clone and sequence full-length cDNAs of *Drosophila Clock*. At the same time, we knew that bHLH-PAS proteins worked as heterodimers; therefore, there should be a partner for CLOCK. Chuck Weitz at Harvard had just successfully found *Drosophila* TIMELESS, the partner of PER, using yeast two-hybrid (Y2H) screening methods. Chuck had made a hamster hypothalamus cDNA Y2H library, and we decided to collaborate to find the partner of CLOCK. In less than a year, we found that a novel bHLH-PAS protein, BMAL1, interacted with CLOCK, and the CLOCK-BMAL1 complex could transactivate the *Drosophila period* gene using reporter assays.[107] The mammalian *Period1* gene had been cloned in September 1997 in both mouse and human, and we tested the upstream promoter region of mouse *Per1* in transactivation assays with CLOCK and BMAL1. The CLOCK-BMAL1 heterodimeric complex was a strong activator of mouse *Per1*; thus, connecting CLOCK and BMAL1 to the *Period1* gene. Steve and I were then able to clone the *Drosophila* ortholog of *Bmal1*. In 1998, at an NSF STC meeting, Rosbash and Hall reported the map positions of two arrhythmic *Drosophila* mutants that they had isolated in screens. Because Steve and I had cloned the *Drosophila* orthologs of mouse *Clock* and *Bmal1*, we knew the map positions of these genes and could see that they mapped to the two arrhythmic *Drosophila* mutants from Brandeis. At an HHMI Science meeting at which Rosbash and I were in attendance,

I revealed to Michael that Steve and I had likely cloned the two Brandeis mutants. We had a conference call with Steve, and we agreed to cooperate and to coordinate our submissions for publication. In addition, Steve asked for the Brandeis *Clock* mutant, which appeared to be a truncation, and in return Michael asked for the *Drosophila Clock* EST sequence that we had determined at Northwestern. Sometime later Michael contacted us and said that we should submit our papers separately. In the end, Weitz and I published the identification of BMAL1 as the heterodimeric partner of CLOCK,[107], and Steve, Chuck, and I published the cloning of *Drosophila dClock* and *dBmal1,* with the additional finding that *Drosophila* PER and TIM fed back on *dClock* and *dBmal1* to repress transcription, thus, closing the feedback loop of the clock mechanism.[108] Rosbash and Hall published two papers in *Cell* reporting the cloning of their two arrhythmic mutants as *dClock* and *cycle* (aka *dBmal1*). These results were all reported verbally in a symposium session at the 1998 Society for Research on Biological Rhythms meeting. The session was stunning because we had solved the initial core elements of the circadian clock mechanism in mammals and flies.

In mammals, the core mechanism included CLOCK and BMAL1 as upstream activators of the *Period* and later *Cryptochrome* (in 1999) genes whose protein products acted as feedback repressors of CLOCK-BMAL1 activation. In 1998, Mike Young's group reported the cloning of the *Drosophila doubletime* mutant, which was encoded by Casein kinase 1. Later in 2000, we reported the cloning of the hamster *tau* gene, which turned out to be *Casein kinase 1 epsilon*.[103] Casein kinase 1 is now known to be a critical component of the clock mechanism that regulates the stability of the PERIOD feedback elements. Thus, by 2000, the conserved core mechanism of the circadian clock in mammals and *Drosophila* was solved.[109]

Ubiquitous Expression of Circadian Clock Genes and Peripheral Oscillators

One of the surprises when we cloned *Clock* in 1997 was that the gene was expressed ubiquitously.[105,110] Our preconceived notion was that all clock genes would be expressed in neurons because the SCN was the locus of the circadian pacemaker in mammals. We now accept that clock genes are housekeeping genes and are expressed in essentially all cells.

Initially, in collaboration with John Hogenesch, Satchin Panda, and Steve Kay, who were working at the Scripps Genomics Institute of the Novartis Foundation (GNF), we used microarray technology to profile circadian gene expression in the SCN and liver of mice.[111] This was the beginning of our ability to observe the impact of the circadian clock system on gene expression in a genome-wide manner and led to the finding that circadian gene expression was largely tissue specific and affected a number of signaling pathways in the SCN and metabolic pathways in the liver.

What was perhaps even more surprising was the observation using circadian gene reporter technology that essentially every peripheral organ system and tissue has the capacity to express autonomous circadian rhythms.[112] This work was pioneered by one my most productive visiting graduate students and subsequent postdoc, Seung-Hee Yoo, who created the PER2::LUCIFERASE fusion gene targeted transgenic mouse in 2004. This mouse line has been distributed to hundreds of laboratories across the globe and has become one of the main circadian reporter mouse lines used in the field. Shin Yamazaki was an essential collaborator in this work because he pioneered the technology for long-term bioluminescence recording in rodent explants in the Menaker lab.

In retrospect, the ubiquitous expression of clock genes is a reflection of the universal capacity for most tissues and cells to express circadian oscillations. These distributed circadian oscillators are cell autonomous and can function independently of the central clock located in the SCN.[112–114] The realization that the body is composed of a multitude of cell-autonomous clocks has raised a number of questions concerning the organization of the clock system and the role of the SCN clock in "circadian organization." My laboratory also contributed to our understanding of the role of the SCN as a master pacemaker to synchronize peripheral oscillators,[112,115–118] as well as the role of intercellular coupling in robustness of the SCN oscillator in work with Caroline Ko, Andrew Liu, Steve Kay, David Welsh, and Danny Forger.[102,117,119,120]

Ethan Buhr, a talented graduate student, explored how the SCN synchronizes peripheral oscillators and discovered that the circadian body temperature rhythm in the mouse is a powerful and universal entraining agent for peripheral oscillators.[117] Interestingly, Ethan found that the SCN is resistant to temperature resetting and found that the intercellular coupling within the SCN accounted for this resistance. When the SCN network was uncoupled with tetrodotoxin treatment, the SCN became sensitive to temperature signals and behaved similar to that seen with peripheral tissues. Ethan went on to show that HSF1 was a mediator of temperature-induced phase shifts and may also be involved in temperature compensation of circadian period. This work and similar work from Ueli Schibler's lab[121,122] demonstrated that subtle circadian rhythms of body temperature can universally entrain all peripheral tissues and thus represents one of the entraining signals that the SCN utilizes to maintain systemwide synchrony.

Mariko Izumo, a talented postdoc, went on to show that conditional knockout of *Bmal1* in the forebrain (BKO) of mice is sufficient to abolish circadian locomotor rhythms, and this led to a desynchrony of the phases of peripheral oscillators in constant darkness.[123] However, exposure of these BKO mice to LD cycles could rescue rhythms and maintain synchrony of peripheral oscillators. When time-restricted feeding (TRF) was imposed on BKO mice in constant darkness, digestive organs such as the liver and kidney

could be entrained by the feeding cycles; however, other tissues became desynchronized and were not influenced by the feeding cycles. Therefore, peripheral circadian oscillators are dichotomous with some requiring SCN signals and others responding to feeding/nutrient cycles.

The discovery of peripheral circadian clocks also led to a deeper understanding of the role of peripheral clocks in organismal physiology. Perhaps the most profound discovery back in 2005 was the finding that the original *Clock* mutant mouse became obese with age and that this was greatly exacerbated by high-fat diet (HFD).[124] Joe Bass and Fred Turek went on to show that *Clock* mutant mice expressed a metabolic syndrome-like phenotype on HFD. This observation, and that from Dan Rudic and Garret FitzGerald,[125] clearly linked circadian mutations in mice with metabolic defects. Since that time, it has become clear that metabolism is one of the primary targets of the circadian clock system.[126,127] My close friend and colleague Joe Bass and I have explored many aspects of circadian metabolism, including the regulation of the NAD+ salvage pathway by CLOCK and BMAL1, and the role of *Bmal1* and *Clock* in glucose and insulin regulation in pancreatic beta cells.[128,129]

Mouse Genetics and Genomics

Between 1997 and 2000, I was invited to be a member of the National Mental Health Advisory Council of the NIMH by the director, Steve Hyman (see volume 10).[130] Two of us on the council were hardcore basic scientists: Richard Scheller[131] and me. Steve Hyman was a strong proponent of using genetics to understand the mechanisms of mental health disorders, and we wrote a consensus working group statement on the "Future of Genetics of Mood Disorders Research."[132]

Harold Varmus was the director of the NIH, and he was a strong proponent of mouse and human genetics and genomics during the early days of the Human Genome Project. I was actively involved in four NIH committees that set priorities for mouse genetics and genomics between 1998 and 2000. It was an exciting time both for human and mouse genome sequencing as well as mouse phenotyping. During this period, the NIH finally began to support ENU mutagenesis projects for neuroscience and behavior, and three large-scale ENU mutagenesis centers were funded to conduct ENU screens for behavioral and physiological phenotypes in the mouse at the Jackson Laboratory, the Tennessee Consortium, and Northwestern University.[133,134]

The overall objective of the Neurogenomics Project at Northwestern was to produce, identify, and distribute mice with new neurobehavioral mutations in five phenotypic domains: learning and memory with Eric Kandel[135] at Columbia, circadian rhythmicity with Turek and me, vision with Larry Pinto and Val Sheffield at Iowa, responses to stress, and responses to psychostimulants with Marc Caron at Duke.[134] New mutations were produced by

chemical mutagenesis using ENU. Our goal was to produce and screen 10,000 mice per year at a rate of 200 mice per week. We developed and scaled these phenotypic tests so that we could achieve 200 mice screened per week. Mice that were isolated as hits in the screens were bred to produce stable mutant lines, and their progeny were phenotypically screened for alterations in these phenotypic domains to confirm transmission of the mutant phenotypes. High-priority mutations were then genetically mapped to low resolution (50–100 meioses). Data and information regarding the mice, and the mutants themselves, were freely available to the wider scientific community. During our five-year screening effort, we isolated more than 200 mutant lines in the five phenotypic domains and dozens of the mutants were cloned. In the vision screen, a number of new mutants were isolated and cloned.[136–139] In the circadian domain, new circadian genes, *Fbxl3* and *Fbxl21*, were isolated, cloned, and mechanistically assigned novel functions within the circadian clock mechanism. FBXL3 and FBXL21 were shown to be novel F-box proteins that defined E3 ubiquitin ligase complexes that target the CRYPTOCHROME proteins for ubiquitylation and degradation by the proteosome complex.[140,141]

At the peak of our screen, our mouse census reached 6,500 cages, and 2,000 cages were required for the ENU mutagenesis and production of 10,000 mutagenized progeny per year. In addition, the breeding and validation of heritability of the hundreds of mutant hits and the mapping of high priority mutants quickly increased our cage census. After about two years into the ENU center award, a new NIMH director, Tom Insel, was appointed, and the ENU centers were not a high priority for him. At the end of our five-year award, the NIMH did not allow us to apply for a renewal. All three centers were faced with figuring out how to continue and how to maintain the hundreds of mutant lines that we had isolated. I was able to obtain a number of NIH awards to support the cloning of circadian mutants and a private foundation grant that Zach Hall arranged from the Zaffaroni Foundation, called the Consortium for Mouse Genetics of Addiction, involving Marc Caron at Duke and Barak Caine at Harvard. I was also able to obtain a Silvio O. Conte Center for Neuroscience Research: Chemical and Genetic Manipulation of Circadian Systems from the NIMH that involved Michael Menaker at UVa; Steve McKnight at University of Texas Southwestern; Carla Green at UVa; John Hogenesch at Penn; and Louis Ptacek from University of California, San Francisco, which helped support the mouse costs along with my HHMI support.

The Allen Brain Institute

In 2002, I was invited to a very interesting event—it was a "charette" organized by Paul Allen to discuss and brainstorm about the possible creation of an institute devoted to the study of the brain. James Watson (CSHL)

and Mark Boguski (NCBI) were the initial advisers to Paul Allen, and the charette took place on one of Allen's yachts (named *Tatoosh*) anchored at Paradise Island in the Bahamas. The venue was quite remarkable. *Tatoosh* was a 303-foot yacht, and I was one of the lucky ones to be able to stay on board for the three days of the meeting. My room was like a Four Seasons hotel room with an Italian marble bathroom. The yacht had a crew of 32 to maintain and operate the vessel. The attendees were Paul Allen, James Watson, Mark Boguski, David Anderson, Cori Bargmann, Mark Adams, Gregor Eichele, Bruce Hamilton, Larry Katz, Marc Tessier-Lavigne, Arthur Toga, Carrolee Barlow, and me. At the time, Allen was one of the top five wealthiest people in the world, and Watson was hoping to convince Allen to donate a $1 billion endowment to fund the new institute. Unfortunately, the dot-com crash had occurred, and Allen decided not to create an endowment but rather to fund the new institute with operating funds. We had wide-ranging discussions on what the institute might do and how it might operate. In the end, the consensus was to create an institute whose initial goal was to map anatomically every gene expressed in the mouse brain and to create a gene expression atlas of the mouse brain that was freely available to all users. Thus, this was the genesis of the Allen Brain Atlas, which was launched in 2003 with a $100 million donation from Paul Allen. I had the privilege of serving on the scientific advisory board (SAB) for the first 10 years (2002–2012) of the institute. Paul Allen was highly engaged with the project and attended all of our SAB meetings. The initial mouse brain atlas was completed in 2007[142] and the Allen Institute has grown and evolved substantially over the years.[143] The model of conducting large-scale projects that could not be achieved in either academia or industry was groundbreaking and has led to innumerable resources for the scientific community.

UT Southwestern

In 2008, returning to my lab and the mouse mutants we had isolated in our ENU mutagenesis center, I was no longer able to maintain these mutant mouse lines at Northwestern given the per diem costs for thousands of mouse cages, and so I began to look for alternative solutions. Shirley Tilghman at Princeton, a prominent mouse geneticist who worked on imprinting, had become the president of Princeton and therefore closed her HHMI lab and her large mouse colony. The empty Princeton mouse colony was the perfect solution for our mice, and I was offered a position as professor at Princeton. I then had to be approved for tenure at Princeton and was asked for letters of recommendation. One of my letter writers was Steve McKnight at UT Southwestern who said, "sure Joe, I'll write you a letter, but I'm going to recruit you too." Steve was the chair of the search committee for the new chair of the Department of Neuroscience, which had been vacated when Tom Sudhof went to Stanford in 2008. In the end, after offers from Princeton,

Penn, and UCSD, I decided to go to UT Southwestern. I had not been looking for a chair position because at Northwestern I was associate chair for many years under Fred Turek and was acting chair for one year when Fred went on sabbatical. That experience taught me that while an interesting position, at Northwestern, it took significant time and effort and this I felt was not good for my research career. Once I became an HHMI investigator, Hughes did not like Investigators to be chairs (with a few exceptions), and so I was able to escape being chair at Northwestern because of this policy. By the time I went to UT Southwestern in 2009, however, there were many HHMI Investigators who also were chairs, including my predecessor, Tom Sudhof. Al Gilman who hired me at UT Southwestern was easily able to get approval for my appointment as chair from HHMI. I went to UT Southwestern for a number of reasons. They solved my mouse situation because per diems were much lower, and I had reduced my colony size. My charge as chair was to double the size of the Neuroscience Department, which included 10 tenure-track faculty and 2 research-track faculty when I began. UT Southwestern had a long tradition of having basic science chairs continuing to be strong scientists and, therefore, they kept administrative duties to a minimum to satisfy the HHMI requirement that we devote 75 percent of our time to research. UT Southwestern has outstanding scientists and by a number of measures (e.g., HHMI investigators, NAS and NAM members) is head and shoulders above Northwestern in the biological sciences. Finally, and perhaps most important, UT Southwestern cares about what you discover, not how much grant money you bring in. This atmosphere and tradition make UT Southwestern an exceptional place to do science.

In my role as chair, I have come to appreciate how rewarding it is to recruit talented junior faculty and to see them grow and prosper. I have recruited and hired 15 tenured or tenure-track faculty into the Department of Neuroscience. So far, all of my junior faculty hires have been promoted with tenure and have gone on to respected careers. These include Tae-Kyung Kim, Gena Konopka, Julian Meeks, Ryan Hibbs, Todd Roberts, Helen Lai, Brad Pfeiffer, Lenora Volk and Wei Xu. Of course, some of my faculty have been recruited away, which is sad for us but a good sign for them as they have gone on to bigger and better places for them.

At UT Southwestern, my lab was able to pursue two new avenues that were difficult to accomplish at Northwestern: (1) next-generation sequencing (NGS), and (2) structural biology. When I moved my lab to UT Southwestern in April 2009, there were no NGS machines on campus. Helen Hobbs and I were able to get the first NGS machine from HHMI instrumentation support. Together with Tae-Kyung (TK) Kim, who came from Mike Greenberg's lab at Harvard and who discovered "enhancer RNAs,"[144] Nobu Koike in my lab used RNA-seq and ChIP-seq methods to perform a comprehensive global analysis of circadian transcription factor binding in the

mouse liver in which all core components of the clock gene pathway were interrogated.[145] In addition, we also analyzed the genome-wide regulation of nascent transcription, RNA polymerase II occupancy, and epigenomic regulation of chromatin by the circadian clock. Although the most robust circadian pre-mRNA cycling occurred at target genes enriched for occupancy of the entire ensemble of core circadian transcriptional regulators, surprisingly, the most pervasive circadian regulation observed on a genome-wide scale were rhythms in RNAPII occupancy, H3K4me3, H3K9ac, and H3K27ac, which occurred at the majority of expressed genes whether or not RNA cycling could be detected. Thus, chromatin modifications associated with RNAPII initiation and transcription were very sensitive indicators of circadian regulation on a genome-wide level.

In addition to the genomics of circadian biology, we were able to solve the crystal structure of the CLOCK:BMAL1 bHLH-PAS heterodimeric complex.[146] By good fortune, when Hans Deisenhofer retired, I was able to recruit one of his senior lab members, Yoga Chelliah, who was an expert in baculovirus expression and purification. Yoga was able to quickly obtain crystals of the CLOCK:BMAL1 complex. The crystal structure revealed an unusual asymmetric heterodimeric conformation of the complex with the three domains of the two subunits, bHLH, PAS-A, and PAS-B, tightly intertwined around each other. Three distinct protein interaction interfaces were revealed in the structure and provided insight into how CLOCK and BMAL1 interacted as well as predicted how these proteins could interact with other regulatory proteins, such as CRY and PER. Thus, the structure of the CLOCK:BMAL1 complex provided insight into the assembly of multi-protein complexes required for circadian function and represented a foundation for decoding the mammalian clock mechanism at an atomic level. Indeed, little was known at the structural level for the entire bHLH-PAS family, which includes 16 members in mammals and is involved in a wide array of functions, including responses to environmental contaminants (Aryl hydrocarbon receptor, AHR), hypoxia (hypoxia inducible factor, HIF), neurogenesis (SIM1), synaptic plasticity (NPAS4), and circadian regulation (CLOCK, NPAS2, BMAL1).[147] In contrast, the structures of modular PAS domains and their interactions with small molecule ligands, such as heme and flavin cofactors, were well understood, especially among microorganisms and lower eukaryotes, where PAS domains serve important roles in two-component signaling and blue-light detection.[148] Although the PAS fold is widely conserved, it has intrinsic flexibility and can adapt to different conformations depending on bound ligands or interacting partners.[149] Despite our understanding of PAS domains and their modular interactions, the intact heterodimeric structure of bHLH-PAS proteins had previously eluded crystallographic solution, perhaps because of their inherent flexibility.[149] Thus, at the time, this was the first crystal structure for any bHLH-PAS transcription factor. The structures for a number of the bHLH-PAS

proteins have now been solved and revealed surprising diversity in heterodimeric interactions among the proteins.[150]

Forward Genetics in Mice: Reprise

Since the initial success of our screen that identified the *Clock* mouse mutant in 1994,[93] I have sought to apply forward genetics in the mouse for gene discovery of other complex behaviors.[151] Because the majority of behaviors that we study are complex at many levels and despite the massive scale of our knowledge of genomic sequence, gene expression, and the rapid use and value of optogenetics to manipulate neural circuits, many behavioral and physiological processes remain a mystery. Genomics and gene editing have made reverse genetics (from gene to phenotype) facile and commonplace, but this approach still requires preexisting knowledge of a relevant pathway or well-supported candidate gene to be effective. I would argue that many interesting behaviors and processes still suffer from incomplete knowledge, and as such, would benefit from an unbiased approach for studying mechanism. This is the power of forward genetics, and in the current era of NGS, classic mutagenesis and screening approaches are having a renaissance.[152] This is true for two primary reasons. First, in mice, loss-of-function alleles are limiting because of lethality (cannot study behavior for lethal genes) and because of paralogous genes (overlapping function). The classic one-third lethal, one-third no phenotype, and one-third with a phenotype still holds true in mouse genetics.[153,154] Thus, despite the existence of the knockout mouse project whose goal was to create null mutations in all mouse genes in ES cells,[153,155] two-thirds of genes are not readily accessible by loss-of-function analysis *in vivo* in mice. This is why point mutagenesis with ENU in mice remains important. More subtle mutants can remain viable and dominant negative mutants can overcome paralogous redundancy. The key to this approach today is that the previously arduous and rate-limiting step of positional cloning has been dramatically accelerated by NGS.

More recently, my lab was able to identify and clone a number of mouse mutants, including circadian F-box proteins (*Fbxl3* and *Fbxl21*), led by Seung-Hee Yoo[140,141] as described previously. In addition, the psychostimulant screen at Northwestern done by a talented postdoc, Vivek Kumar, led to the identification of *Cyfip2* as a gene that reduces the hyperlocomotion response to cocaine in mice.[156,157] Importantly, Vivek found that a missense mutation in *Cyfip2* occurs in the C57BL/6N substrain from NIH and is also found in C57BL/6 substrains distributed by Charles River (C57BL/6NCrl) and Taconic (C57BL/6NTac) in the United States.[157] Thus, caution should be exercised when using different C57BL/6 mouse strains because we and others have found that there are more than 50 genes carrying sequence variants that are likely to have functional consequences in C57BL/6N compared

with C57BL/6J (the gold standard mouse strain and genome reference mouse strain).[157,158]

The most difficult phenotype for gene identification in our behavioral screens was context-dependent fear conditioning. As I mentioned earlier, the coefficient of variation for the freezing score in fear conditioning was a whopping 40 percent, which meant that individual mice could not be reliably scored as mutant or WT in this test. Therefore, the mutants that we isolated in the recessive screen were dominant mutations that could affect the entire pedigree quantitatively. A second problem was that mapping crosses to conventional inbred strains revealed quantitative trait locus (QTL) effects that increased the variance of the assay and masked the effects of the original mutant. Thus, it was not until an extremely persistent and rigorous postdoc, Pin Xu, took over the task of cloning the underlying genes involved that we succeeded in cloning fear conditioning genes. First to overcome the QTL effects of inbred strains, Pin used a closely related strain, C57BL/10J, to create mapping crosses with the mutant, which was on a C57BL/6J background. This greatly reduced the masking effects of QTLs and allowed mapping crosses to work. The first mutant that Pin identified was a line that exhibited very low freezing scores. Pin was able to show that this mutant was deficient in learning or encoding, and thus we named the mutant, *Clueless*. Eventually Pin was able to show that *Clueless* was caused by a point mutation in the potassium channel, *Kcnc3*,[91] leading to a very strong loss-of-function in KCNC3 conductance. KCNC3 is highly expressed in dentate granule cells of the hippocampus, and thus, the likely site of action of the mutation occurs at this anatomical locus.

Perhaps one of the most demanding screens that we conducted was in a collaboration with Masashi Yanagisawa at UT Southwestern to find sleep genes. About two decades earlier at a biotech startup named Hypnion, which was initially founded by Emmanuel Mignot, Michael Rosbash, and myself, one of the goals was to identify sleep genes using ENU mutagenesis and EEG phenotyping. Unfortunately, the ENU screen was never accomplished at Hypnion. When I relocated to UT Southwestern, however, Masashi and I decided to do the screen. One of the reasons no had done such a screen was the massive effort involved. Each mutagenized mouse would have to be stereotaxically implanted with cranial electrodes, recover, and then sleep recorded by EEG for three days. The advantage of the EEG screen was that the phenotype was robust and quantitative, and Masashi's lab demonstrated that most of the EEG phenotypes possessed low coefficients of variation so that we could isolate variants that were more than 3 standard deviations from the mean (or a *Z*-score >3.0). Using a dominant screen, we knew that it might require screening many thousands of mice to be successful. Masashi was undeterred by these numbers and developed methods to scale up the surgeries and EEG recordings. Initially at UT Southwestern, Masashi's lab could screen 32 mice per week. Later, he doubled this capacity

when he relocated to Japan to found and direct an institute devoted to the study of sleep at the University of Tsukuba. We published the ENU screen for sleep mutants in 2016 and described the first two mutants from the screen named *Sleepy* and *Dreamless*.[159] *Sleepy*, whose phenotype was excessive sleep time and sleep need, was caused by a point mutation in the splice donor site of exon 13 in the *Sik3* gene, leading to an internal deletion of 52 amino acids in the SIK3 protein. Masashi and his colleague have gone on to show that SIK3 phosphorylation and HDAC4 acting in excitatory neurons regulate sleep need or drive, which is the homeostatic process underlying the process of sleep. The second mutant, *Dreamless*, whose phenotype was short REM episode duration, was caused by missense mutation in the *Nacln* gene.[159] NACLN is a sodium leak channel that leads to increased excitability of neurons in the brainstem that control the REM-off state. This work showed that despite decades of work on sleep mechanisms, forward genetics was able to reveal an entirely new pathway, SIK3, that regulated the homeostatic control of sleep and sleep need.

Circadian Rhythms and Parasites

A completely new area of research in my lab was pioneered Filipa Rijo-Ferreira who was initially a visiting graduate student in the lab. Filipa was the lynchpin forging a novel collaboration between my circadian laboratory at the UT Southwestern and the laboratory of Luisa Figueiredo at the Instituto de Medicina Molecular in Lisbon, Portugal. Filipa was a graduate student in the GABBA Program, a competitive doctoral program in the areas of basic and applied biology in Portugal. Filipa, under her own initiative, proposed to study *Trypanosome brucei* and circadian clocks for her graduate work. Because Luisa worked in the area of immunology, she thought that they would need more expertise in circadian rhythms; therefore, Filipa visited a number of circadian laboratories in the United States before selecting mine as the one with which she would like to collaborate. This was the beginning of one of the most interesting and productive collaborations that I have undertaken because it opened a new direction of research, and the discoveries that Filipa made were novel and unexpected.[160]

In collaboration with my lab, Filipa pursued a project during her graduate studies on whether *T. brucei*, itself, has an endogenous circadian clock system. In Luisa's lab, Filipa used RNA-seq to profile gene expression in cultured *T. brucei* that were synchronized by 24-hour temperature cycles and sampled over two circadian cycles. Filipa examined both bloodstream and insect forms of the *T. brucei* parasite. She found hundreds of RNA transcripts that expressed circadian oscillations, and these oscillations were completely independent of the cell cycle in these parasites (which had a cell division cycle period of about 7 hours). Thus, the trypanosome population expresses a circadian oscillation that transcends the cell division cycle.

She found two major phase clusters of cycling genes, and these genes were enriched in a number of metabolic pathways. She then tested whether *T. brucei* exhibited a time-dependent circadian sensitivity to either metabolic insults (redox stress) or the drug suramin. She found in both cases that there was a circadian sensitivity to redox stress and mortality with suramin treatment. This provided evidence that circadian rhythms are physiologically significant in *T. brucei*.

In my lab, Filipa made two major discoveries in two areas of circadian biology and parasites. First, as a postdoctoral researcher in my lab, Filipa proceeded to address her original project on the effect of *T. brucei* infection on circadian rhythms and sleep in the mouse. Filipa discovered that *T. brucei* infection of mice not only induces characteristics of sleep sickness, but also has profound effects on the circadian system of mice. The regulation of sleep and circadian rhythms are related and linked, but each can be mechanistically separated at the molecular and brain circuit levels. In her initial work, Filipa found that as *T. brucei* infection progressed, the infected tissues displayed both an earlier circadian phase and a shortened circadian period. This occurred first in adipose tissue and then eventually in the brain and SCN, the site of the master circadian pacemaker in mammals. At the late stages of infection involving the brain, the behavior of the mice became more diurnal with an abnormally early phase of activity relative to uninfected controls that remained nocturnal. Body temperature circadian rhythms were also similarly affected. Thus, *T. brucei* infection clearly targets the circadian system as well as the sleep regulatory system. Thus, we proposed that "sleeping sickness" is actually a circadian disorder of the timing of sleep, which manifests in an excess of inappropriate sleep during the normal waking hours and a decrease in sleep during the normal sleeping hours of the day.

Second, Filipa delved into a new parasitic infection—malaria.[161] She discovered that the periodic fevers that are characteristic of this disease are the result of the intrinsic clock in the *Plasmodium* parasite. We were intrigued by the fact that fevers occur every 24, 48, or 72 hours, depending on the species of *Plasmodium*, which means they are all multiples of 24 hours, or "modulo-24," which is a signature of an underlying circadian process. (A well-known example of a modulo-24-hour process is the rodent estrous cycle, which is typically four to five days in length in mice, but the timing of ovulation is gated by the circadian clock.) The rhythmic aspect of this infection has been known for a long time, but the prevailing view in the field is that this was driven by the *Plasmodium* cell cycle and more recently by the host's circadian rhythms. Through a series of elegant experiments Filipa dissected the different possible confounds, and she was able to show that the parasite population requires host cues to synchronize to each other but that the rhythms themselves are driven by an intrinsic parasite clock.

*Importance of Circadian Time in Metabolic Function, Aging,
and Longevity*

Related to our work on the role of central vs. peripheral circadian oscillators,[162] we found that peripheral tissue clocks are differentially synchronized by light cycles acting centrally as compared with nutrient cycles imposed by TRF.[123] When the light-dark cycle and the time of feeding are in conflict with their normal phase relationship, the brain and many peripheral organs remain locked to the light cycle, whereas "metabolic tissues," such as the liver and kidney, can be reset by feeding cycles.[123,163,164] In addition to the entrainment of peripheral tissues by nutrient cycles, the timing of nutrient consumption has dramatic effects on metabolism. For example, my colleague at Northwestern, Fred Turek, showed that restricting an HFD in mice to either 12 hours during the daytime or 12 hours during the nighttime had completely different effects on body weight.[165] The mice that ate HFD at the wrong time during the day became obese, whereas the mice that ate the same HFD at the correct time at night were protected from weight gain. These types of TRF experiments have been replicated by many groups and restriction of overnutrition diets to the active night phase protects against weight gain and metabolic health in mice.[166,167] This work has been translated to humans, and many studies have shown that time-restricted eating (TRE) in which nutrient consumption is restricted to 8- to 10-hour windows during the awake active phase has beneficial effects on weight loss, glucose regulation, and other health measures in people.[168] Thus, the timing of food consumption relative to the phase the circadian system in both mice and humans has important consequences on metabolism, body weight, and health.

Having become interested in TRF experiments and their health benefits in rodents, we wondered whether TRF might also have beneficial effects on aging. In the aging field, it has been known that caloric restriction (CR), a reduction in caloric intake without malnutrition, is the most effective nonpharmacological intervention for extending lifespan in model organisms.[169,170] For example, a 30 percent reduction in calories leads to a 30 percent extension of lifespan in mice. However, despite the fact that this beneficial effect of CR was first reported more than 80 years ago,[171,172] the underlying mechanisms are still largely unknown.

An extremely talented postdoc, Victoria (Vicky) Acosta-Rodriguez, took on this new project. We found profound effects of CR on the behavior of mice, and these effects raised some questions concerning the interpretation of classic CR experiments on longevity.[173] In these classic CR experiments, rodents were typically fed intermittently on Mondays, Wednesdays, and Fridays (for logistical reasons).[174] In addition, the food was given in the morning, a time when nocturnal rodents do not normally consume most of their food. As described earlier, timed food administration is a potent signal

that entrains circadian clocks, particularly those in peripheral tissues, such as liver.[175,176] Thus, in addition to reducing daily energy intake, CR resets complex circadian programs of gene expression in tissues throughout the body. Although the reduction in energy intake is commonly thought to be the critical factor that extends lifespan, it is possible that the timing of the food intake is a key component. For example, it is well known that high-fat diet (HFD) leads to obesity in mice. As described earlier, if the HFD is restricted to the nighttime, then mice do not gain weight.[166] In contrast, if the same HFD is given only during the daytime, then the mice become obese.[165] Thus, the obesogenic effect of HFD is caused primarily by consumption at the wrong time of the circadian cycle.

Once we began to contemplate conducting longevity studies on caloric- and time-restriction in mice, it became clear why previous CR longevity experiments used protocols in which the mice were not fed on a daily basis—these experiments could last as long as four years to complete, and it is a logistical challenge to feed the mice every single day without fail for the duration of the study. TRF experiments, if conducted manually, would require twice as much labor because the food would have to be given and taken away each day. Thus, to study the importance of time vs. calories, we developed our own automated feeding system.[173] We built our own automated feeders for two primary reasons: commercial products are too large, are expensive, and cannot be integrated with our circadian activity system. In my lab, we can record activity rhythms from more than 2,000 singly housed mice, and we have now added more than 1,500 automated feeders to our activity cages. This scale is required because ideally mice must be individually housed for longevity experiments to have continuous feeding and activity recordings, and as described earlier, the experiments take three to four years to complete, and large numbers of mice (>36–96 per condition) are required for Kaplan-Meier survival analysis.

Our automated feeding system revealed that different feeding schedules induced specific temporal patterns of food intake. We found that CR groups consumed their food within 2 hours regardless of the time of food availability (day or night) even though they had 24 hours of food access. This severe self-imposed TRF pattern suggested that a circadian component could contribute to the beneficial effects of CR.[177] Although a reduction in energy intake is commonly thought to be the critical factor that extends lifespan and well-being in the CR regimen, it is possible that the timing of the food intake is also a key component. Indeed, we found a profound difference in lifespan in C57BL/6J male mice that were calorically restricted to the same extent (30 percent CR), but that were allowed to consume food each day either immediately within 2 hours (self-imposed) or spread out over 24 hours. Surprisingly, the CR-spread fed mice had only a 10 percent longer median lifespan than ad libitum fed mice. Introducing a fasting interval but eating at the wrong circadian phase (CR-day fed) led to a 20 percent

extension in median lifespan; in contrast, mice with circadian alignment of feeding (CR-night fed) had a *35 percent extension of median lifespan*.[177] Thus, calorie reduction alone cannot lead to the dramatic extension of lifespan seen historically unless fasting and circadian alignment of feeding time are present.

Human studies are beginning to support the hypothesis that restricting the time of food consumption each day has beneficial effects.[167] Human studies also show that substantial degrees of CR (e.g., 30 percent) cannot be achieved by the majority of subjects for the prolonged times (decades?) necessary to extend lifespan.[178] Thus, TRE may be a more practical way to achieve health benefits in people.[168] We believe that our work will have the potential to affect the health of people in a direct and significant way.

Texas and Colorado

Having moved from Northwestern University to UT Southwestern in 2009, a lot of changes occurred in my life. Sadly, I separated from my first wife, Barbara, and later divorced. When I first split with my wife in 2008, Joe Bass took me in and I lived with Joe in his house until I moved to Dallas in 2009. Joe was also divorced so it was truly a wonderful time for the two of us to spend time drinking coffee in the mornings and having dinner and wine in the evenings discussing science, politics, and life. I will always be grateful to Joe for giving me a safe place to rest and recover. Dallas was very different from Chicago, but both cities are surprisingly ethnically diverse. The food scene in Dallas is very strong and interesting. Being a foodie, I was pleasantly surprised to find that Dallas has extremely good Japanese restaurants owned and run by Japanese chefs.

Once I moved to Dallas, I was able to recruit one of my circadian colleagues, Carla Green, from UVa. Our labs work closely together on many aspects of circadian biology. I was able to recruit a third circadian colleague, Shin Yamazaki, from Vanderbilt University in 2013. The three labs form a circadian core group, and we have a joint weekly/biweekly lab meetings together. Our UT Southwestern circadian group then became members of the Texas Society for Circadian Biology and Medicine, which meets annually at the three major circadian hubs in the state at Texas A&M, UT Health Houston, and UT Southwestern. Carla Green and I were friends for a long time, and in Dallas, we began to spend time together. In 2019, we were married. We both loved to play tennis and ski and like to go on vacation in Colorado for both skiing and hiking. Our favorite place in Colorado is the Roaring Fork Valley where Aspen, Snowmass, Basalt, Carbondale, and Glenwood Springs are located. Hiking in the "valley" is simply stupendous. Every valley/river/creek flowing into the Roaring Fork River is beautiful, but in addition, each is also unique in its terrain and flora. There is Independence Pass with its high alpine terrain; Castle Creek, Maroon Creek, and Snowmass Creek with

their abundant aspen, fir, and spruce trees; the Frying Pan River valley with it red rocks and mixed coniferous forest; Sopris Creek valley, which is drier, with Gambel oak, piñon pine, and juniper forests interwoven with sagebrush meadows; and the Crystal River valley, a mix of both Gambel oak and mixed coniferous forest. Glenwood Canyon and the hot springs in Glenwood Springs cap off the end of the valley. By a stroke of good fortune, we stumbled upon a beautiful property overlooking Mount Sopris in Basalt along West Sopris Creek Road. It is a unique find because it is located in a relatively undeveloped valley so that it feels remote, yet it is only 15 minutes away from old town Basalt in one direction and 15 minutes away from two grocery stores and a modern complex in the other direction. In addition, Aspen is only 20 miles away with all its culture, art, and music. Finally, there are five ski resorts within a 30-minute drive. We plan on building a house there and eventually retiring there. I have always wanted to have a season ski pass and be able to look outside and decide if I wanted to go skiing that day or not.

Gratitude

In closing, throughout my career I have been blessed with exceptional mentors, students, postdocs, colleagues, collaborators (too many to have described here), and supportive institutions (Northwestern, HHMI, and UT Southwestern). In addition to my mentors who I have already mentioned (Ken Rawson, Pat DeCoursey, Mike Menaker), I am grateful for the support that I received from Colin Pittendrigh and Jurgen Aschoff who founded the field of circadian rhythms and who I was fortunate to interact with. Special thanks are due to my friend and colleague Fred Turek, at Northwestern, who recruited me there and helped launch my academic career. I feel extremely fortunate to have been selected as an HHMI Investigator, and my association with HHMI was clearly essential for taking my career to new levels. During my journey, I was very fortunate to have met and interacted with Eric Kandel (see volume 9),[135] Zach Hall, Floyd Bloom (see volume 7), and Solomon Snyder (see volume 6)[179] all of whom were very supportive of my career, including inviting me to serve on scientific advisory boards (e.g., Eric Kandel: Bristol-Myers Squibb Neuroscience Award Selection Committee; The Klingenstein Fund, Neuroscience Advisory Committee) or nominating me to the National Academy of Sciences (Sol Snyder). In the field of mouse genetics, Bill Dove, Wayne Frankel, Jeff Friedman, Eric Lander, Allan Bradley (HHMI and Sanger), and Steve Brown (Harwell) as well as many colleagues at the Jackson Laboratory were very supportive and welcoming to me as I entered my second career as a mouse geneticist. At UT Southwestern, Steve McKnight, Al Gilman, Dan Podolsky, and David Mangelsdorf have given me unwavering support. In the molecular genetics of circadian clocks, Michael Rosbash, Mike Young, and Ueli Schibler have been my close friends

and colleagues for decades as we navigated the path to where we are today. Finally, I want to thank my wife Carla, my close friend Joe Bass, and my family (especially my son Matthew and my daughter Erika) for putting up with me through the years and having such a good time!

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