Neuroblastoma Research, 1967-1976

Marshall W. Nirenberg is best known for his work on deciphering the genetic code by discovering the unique code words for the twenty major amino acids that make-up DNA, for which he won the Nobel Prize in Medicine or Physiology in 1968.

Nirenberg was the first government scientist to win the Nobel Prize. The National Library of Medicine and the Office of NIH History has amassed a collection of correspondence, laboratory administrative and research materials, and publications that documents Nirenberg's career as a researcher in biochemical genetics at the National Institutes of Health.

Dr. Nirenberg is featured in The Profiles in Science web site of the National Library of Medicine celebrates twentieth-century leaders in biomedical research and public health. Students appreciate the history, and share some of the excitement of early scientific discoveries in molecular biology. The National Library of Medicine is digitizing and making available over the World Wide Web a selection of the Marshall W. Nirenberg Papers, for use by educators and researchers.

In 2007, the Archives and Modern Manuscripts Program, History of Medicine Division completed a Finding Aid to the Marshall W. Nirenberg Papers, 1937-2003 (bulk 1957-1997). Individuals interested in conducting research in the Marshall W. Nirenberg Papers are invited to contact the National Library of Medicine.

The NLM digital materials and references provide the background for the series of six interviews conducted with Marshall W. Nirenberg, Ph.D., by Ruth Roy Harris, Ph.D., between September 20, 1995 and January 24, 1996.

The "Harris Interviews" took place in Nirenberg's laboratory on the campus of the National Institutes of Health (NIH) in Bethesda, Maryland. Harris also conducted several supplemental interviews, both by telephone and in person, with individuals either involved in the breaking of the genetic code or personally acquainted with Nirenberg: James Pittman, Joan Geiger, Philip Leder, Thomas Caskey, Sidney Udenfriend, and Perola Nirenberg. Interviews with Pittman and Geiger are now in the Marshall Nirenberg Collection at the National Library of Medicine (NLM). Notes from other interviews are held at the Office of NIH History.

A number of individuals and institutions worked on editing the interviews for clarity and content: Sarah Leavitt, Victoria Harden, Caroline Hannaway, Alan Schechter, Robert Balaban, and Alan Peterkofsky. Caroline Leake, Katrina Blair, and Mary Alvarez provided administrative and technical assistance. In 2008, Deborah Kraut edited and formatted the interviews to correspond to the NLM digital materials.

Each Section begins with the NLM digital summaries summaries and references. Additional references, when appropriate are added:

From NLM Profiles in Science:

http://profiles.nlm.nih.gov/JJ/Views/Exhibit/narrative/neuroblastoma.html

Marshall Nirenberg's curiosity was piqued by neuroblastomas, which are malignant tumors composed of developing neurons. Fully developed neurons differentiate into specific cells to perform specific tasks, so scientists cannot use the adult neurons to study neural development because they no longer divide. Nirenberg became interested in neuroblastomas because he thought the tumor neurons might retain the properties of differentiated neurons. He could then use them as a model system to study the development of neurons. The neuroblastoma system also offered Nirenberg an opportunity to study neurotransmitters in the brain. Neural information is communicated between neurons by way of chemical neurotransmitters such as norepinephrine and dopamine. Studying neurotransmitter synthesis with the neuroblastoma system enabled Nirenberg to investigate the details of information processing in the nervous system in much the same way that the study of protein synthesis enabled him to explore the details of information processing in the genetic system.

In the 1960s, scientists typically studied neuroblastomas only to fight the cancerous cell growths, which most often afflict infants and young children. Nirenberg made an innovative move by using the tumor cells towards a different end--he wanted to study the formation of neurons in tissue culture. Tissue cultures are small amounts of undifferentiated tissues or single cells grown in an artificial environment such as a petri dish. Nirenberg's study of neuroblastoma was one of the first times neurobiologists used tissue cultures, an experimental method that now pervades the field. The innovative use of tissue cultures forced Nirenberg to learn new experimental methods. "I had never done any tissue culture research before," Nirenberg remembered in a 2001 interview, "I just jumped in with feet, hands, and everything." Nirenberg enlisted the help of a fellow colleague at the NIH, Dr. Phillip Nelson, an expert neurophysiologist who taught Nirenberg the new methods needed to analyze the neuroblastoma tissue cultures. In turn, Nirenberg taught Nelson the biology needed to study the molecular components of the research.

Nirenberg and Nelson's combined approach to tissue cultures allowed for the neuroblastoma cells to develop in vitro. With the in vitro method neurobiologists could remove the neuroblastoma cells from the original tumor, grow the cells on petri dishes, and then study them with a microscope while they were still developing. The neurons could then be observed and studied in this isolated state. As Nirenberg and Nelson mastered the neuroblastoma system, they were able to clone and grow tumor cells based on the neural properties of the neuroblastomas, thereby creating cell lines of tissue cultures with desired characteristics. Nirenberg could develop specific cell lines based on characteristic traits such as how fast the neurons grew, how receptive the neurons were to morphine, or how the neurons synthesized a given neurotransmitter. Nirenberg and Nelson farmed a variety of different cell lines and even created a cell bank to store the various strains. Scientists all over the world still request samples of these cell lines from Nirenberg's laboratory.

The cell lines were used for multiple projects. For example, Nirenberg, with the help of post-doctoral fellow Xandra O. Breakefield, developed an experimental method that could

distinguish neurons from the neuroblastoma system based on their abilities to synthesize different neurotransmitters. This method let Nirenberg and Breakefield classify the various cell lines formed from neuroblastomas even more precisely. Although Breakefield worked with Nirenberg at the NIH in the 1970s, this formal collaboration was not their first encounter. Breakefield initially contacted Nirenberg in 1962 when she was a sophomore at Wilson College in Chambersburg, Pennsylvania. Interested in biology, Breakefield sought career advice from the biochemist who had just deciphered the first word of the genetic code. Breakefield even visited Nirenberg's laboratory in 1962 to watch science in action. A decade later, Breakefield joined Nirenberg's laboratory as a post-doctoral fellow.

In the early 1970s, Nirenberg also utilized the neuroblastoma system to study the effects of morphine on the nervous system. Werner Klee, a biochemist at the National Institute of Mental Health at NIH, was interested in the chemical effects of morphine on the brain. Nirenberg and Klee developed a neuroblastoma cell line that possessed an unusually high percentage of morphine receptors. They found that morphine inhibits the production of an enzyme in the brain called adenylate cyclase, which promotes the construction of complex molecules vital to neural transmission. To counterbalance the reduction of adenylate cyclase, Nirenberg and Klee discovered, the brain then overproduces the chemical. If the morphine stimulation is removed, the increased level of adenylate cyclase production causes the brain to be flooded with this chemical. Nirenberg and Klee found that an individual who experiences this chemical imbalance goes through a withdrawal period as the nervous system slowly returns the adenylate cyclase production to its proper level.

Nirenberg studied neuroblastomas for more than a decade. The model system offered him a versatile tool to explore the intricate details of the nervous system. Most importantly, the neuroblastoma system allowed Nirenberg to use many of the conceptual and methodological practices he developed from biochemistry and molecular biology in a completely new field of research.

The Harris Interviews – 1995 – 1996

Marshall Nirenberg (MN): When I went in ***to this field***, I was a real pioneer. There is no question about ***that.*** ~~my being a pioneer in the field~~. I finally decided to study neuroblastoma cells, which are neuroblast tumor cells. Normal neurons don't divide whereas the tumor of neurons continuously divides. I decided to establish clones—of neuroblastoma cells and characterize them. I thought that they would express many of the same genes that normal neurons would

express in the nervous system. My long-range objective was to try to establish clones that were able to form synapses with other cells and use this as a model system in which to study information processing in the nervous system.

John Minna [a post-doc] played a major role in generating somatic hybrid cell lines by fusion of mouse neuroblastoma cells with other cell types, clonal cell types, and with embryonic cells from the nervous system and establishing clones. He was smart, fast-moving, and very effective in doing research. At that time, we were establishing somatic hybrid cell lines, fusing two cells together and selecting their progeny. That alters the gene expression in these cells, which we then could use to determine whether we had turned on genes for neuroproperties. He was very effective in generating hybrid cell lines.[1] He did a tremendous amount of work on this, and we published a major paper. This was the first time anything like that had been done. We did this in a very crude way to change gene expression, to rescue gene expression from neurons or other cell types in the nervous system, and it worked quite well. That was his major contribution. Minna stayed on as an independent investigator and was a section head in my lab before he left to become head of a big laboratory in the Cancer Institute here with clinical responsibilities.

Xandra Breakfield,[2] a very interesting young woman, first came to visit me when she was an undergraduate in school, and she heard that we were doing some interesting work on the code. She made an appointment with me and on a Christmas vacation or spring break came to talk to me, to find out what was going on, which was very unusual. She is the only person whom I can remember talking to about this. Then she went on to get a

Ph.D. after her undergraduate work. She got a Ph.D. in microbial genetics at Georgetown, and then she came to do postdoctoral work with me.

Her background was terrific for the work that I was starting in the neuroblastoma work. She did a beautiful project with me. She worked out a way of selecting for adrenergic neuroblastoma cells. It worked beautifully. We could select one cell out of 75,000 cells that made catecholamines. It was an adrenergic cell. Tyrosine is one of the amino acids that is required. Cells require tyrosine for survival. So we grew the cells in the presence of phenylalanine but not tyrosine. Phenylalanine can be converted to tyrosine by hydroxylation, which is catalyzed by tyrosine hydroxylase, by an enzyme that would hydroxylate phenylalanine and enable the cells to survive if they had this enzyme. We used this approach: it was an extraordinarily effective selection method for selecting for rare cells that expressed the gene for tyrosine hydroxylase. Xandra has gone on to win awards in neurobiology.

She now is director of a laboratory of neurobiology in Boston. I think it is associated with the Massachusetts General Hospital or Harvard. At one time she was the director of the Eunice Kennedy Institute in Boston or near Boston. She has done excellent work. She has won all kinds of prizes, and I am very proud of her. She is an alumna of the laboratory.

I have worked with some extraordinarily talented people, and that has been a wonderful and ongoing source of pleasure. Early on I became friends with Philip Nelson here at the

NIH, an expert neurophysiologist and head of a group of neurophysiology. We collaborated, and it was a mutual and productive collaboration. I had to learn his language, and he had to learn mine. He taught me neurophysiology: I taught him molecular biology. He recommended books for me to read and answered questions. We each, I think, became better scientists because of the collaboration. We collaborated with Phil Nelson in characterizing the electrical channels, and these hybrid cell lines proved to be extremely useful for later studies.

[William] Bill Catterall, in fact, used many of these cell lines in his work in characterizing the ion channels He came as a postdoctoral fellow and was interested in studying ion transport, and that is what he did with the neuroblastoma cells. Then, he worked as an independent investigator for a number of years in the lab, and now he is chairman of the department of pharmacology at the University of Washington at Seattle. An excellent scientist, one of the best people I have ever worked with.[3] When [William] Bill Catterall came to the lab, he was very much interested in characterizing ion channels and went ahead and did superb work in characterizing the sodium channel of the neuroblastoma cells. He has built quite a well-deserved reputation for himself for the superb, beautifully clean work that he has done with ion channel proteins, which he isolated later and utagenized. He determined the effects of mutation and correlated the amino acid substitutions with the electrophysiologic consequences of those changes in amino acid substitution in the protein, beautifully elegant work. He also has won many awards in neurobiology for the work that he did after he left the lab.

We found that many of the neuroblastoma cells that we had cloned were capable of generating action potentials — that electrically-excitable membranes were inducible, that ion channels were inducible, and that electrically-excitable ion channels were inducible.

Ultimately, we found that we could shift populations of cells from a relatively undifferentiated state to a neuronal differentiated state by treating the cells for long periods of time with compounds that elevated intracellular cyclic-AMP levels. Then, they expressed these ion channels. They acquired electrically-excitable membranes: they had voltage-sensitive sodium channels, voltage-sensitive potassium channels of several kinds, and several types of voltage-sensitive calcium channels as well. Ultimately we found cell lines that could form synapses *in vitro* with clonal muscle cells, striated muscle cells. Takehiko Amano, who came here as a postdoctoral fellow, was a tremendous help in establishing the clones. He had a green thumb when it came to cell culture, and he established many of the clones that we worked on and later characterized.

I then started to work with a number of postdoctoral fellows. At the time that I went into this cell culture work—up to perhaps a year before I went into this field—the predominant view was that if you cultured cells *in vitro*, they dedifferentiated and that you could not culture differentiated cells.

Nevertheless, about a year or so before someone published a paper showing for the first time that you could, in fact, culture differentiated cells that retained differentiated properties. So my work began at a very early stage in understanding cells of the nervous system.

Although cells from the nervous system had been cultured in explants as many as 50 years earlier, nobody had tried to establish clonal cell lines from the nervous system and

characterize these cell lines. We needed assays. We could clone many of the cell lines, but we had to find out by assays what we had.

We established a whole battery of assays. We assayed enzymes that catalyzed neurotransmitter synthesis or breakdown, ion channels of various kinds, and it took several years of intense work by perhaps four or five of the postdoctoral fellows.

Roger Rosenberg is a neurologist, and he had had very little experience in the laboratory when he came here. He was a research associate but learned all of the stuff that we were doing with neuroblastoma cells. He is chairman of the department of neurology at Southwestern Medical School in Dallas, Texas.

So, we were definitely pioneers. But, at least we established many hundreds of cell lines. We worked with 26 or 27 of the cell lines (the major ones) that we characterized to change gene expression in a very crude, gross way. We made somatic cell hybrids. We fused the neuroblastoma cells, with other cell lines and then we would get a single cell with two nuclei. The nuclei would fuse to form one nucleus, and they would be in an unstable state and the chromosomes would be discarded in different combinations. So we would end up with cell types that had different genotypes and would express different sets of genes. We characterized many of these somatic hybrid cells and found that in some cases we rescued gene expression. We fused neuroblastoma cells with normal embryonic cells from the normal nervous system and rescued, I believe, gene expression from the normal cells. We also lost gene expression. We generated, in essence, mutants

that were defective in various ways with respect to forming synapses with other cells. Eventually we found four to five neuroblastoma cell lines that were able to form synapses with striated muscle cells. These synapses were regulated. We could innervate every muscle cell on the plate, and it was clear that many of the properties that we were studying involved regulation of gene expression.

My objective was to generate novel, simple cell systems that could be used as model systems to study properties of the nervous system, and we used them in many different ways. For example, early on in a collaborative effort with Werner Klee we looked for opiate receptors and found that some of the cell lines had many opiate receptors. We initiated a whole series of work with Shail Sharma, who was visiting on a sabbatical from India.

We found that if you culture cells in the presence of morphine, the morphine inhibited adenylate cyclase activity. We observed that if you continued to culture the cells in morphine, they become tolerant to the morphine with respect to adenylate cyclase activity. First, you get this marked inhibition of adenylate cyclase that would then slowly, gradually, come back to the normal level. But when you withdrew the morphine, then you saw that what had happened was that much more adenylate cyclase activity had formed during this period of acquiring tolerance to morphine. When you withdrew it, levels of adenylate cyclase became extremely high, and that the morphine was just an inhibitor of adenylate cyclase. It looked as though the normal level of adenylate cyclase was present. But, in fact, the level of adenylate cyclase activity was much higher than

normal, and it could be unmasked by withdrawing the morphine inhibitor. So this provided a simple molecular explanation for complicated processes involving addiction and withdrawal and tolerance and withdrawal to opiates. We characterized these receptors and this phenomenon and studied it in various ways. In fact, we used these cell lines as model systems to study different aspects of the nervous system. We found many species of receptors that were present and characterized many of the cell lines.

Haruhiro Sugiyama,Ph.D. came from Japan and worked on muscarinic acetylcholine receptors in the developing retina. Rick McGee and Paul Simpson also were postdoctoral fellows who worked on regulation of acetylcholine release from neuroblastoma-glioma hybrid cells.

Neal Nathanson, then a postdoc, shed light on the addiction process. Neal showed that muscarinic activators, like carbachol, could result in a decrease in adenylate cyclase activity. He found that treating the cells with muscarinic acetylcholine receptors with an agonist, with an activator of the receptor like carbachol--it's a stable activator--would have similar effects that resembled opiates. Consequently, the activation of a muscarinic acetylcholine receptor would inhibit adenylate cyclase activity when you add carbachol. Well, carbachol is an agonist, and it would reduce adenylate cyclase activity. And if you cultured cells in the presence of carbachol, cells that had the appropriate species of receptor-- of muscarinic acetylcholine receptor--you would see a marked inhibition, of adenylate cyclase activity. Then slowly, gradually, over a period of hours, the cyclic AMP levels of cells would gradually return to normal.

That was because the activity of adenylate cyclase increased quite markedly so that the cells basically would then become dependent on carbachol. If you removed carbachol, you unmasked the relatively high activity of adenylate cyclase compared to control cells, and the cells had too much activity of adenylate cyclase activity. This was very similar to the phenomenon that we had described with morphine, with opiates. The cells cultured in the presence of an opiate become dependent on it to continue to inhibit adenylate cyclase. That is because the adenylate cyclase--the level of cyclic AMP and of adenylate cyclase activity--gradually comes back to the normal level in the presence of the inhibitor. If you remove the inhibitor, you unmask the relatively high activity, and that's very much akin to the withdrawal affect dependence. We see dependence on a compound and tolerance to the compound, and it's very similar to the addiction process. I think it is the addiction process that we found, a mechanism of addiction, even though we still don't know some things about it. But we proposed this as the method.

I wouldn't go so far as to say in public that we studied addiction because we were studying cells, not animals; but the same thing happens with intact animals. Other people have shown this, and other people have confirmed our findings. Today, even, some 20 years after we first published this, this is still the preferred mechanism for the understanding of opiate dependence and tolerance and withdrawal.

Steven L. Sabol, first came to the lab as a postdoctoral fellow, then as a research associate. He had obtained the Ph.D. degree with Severo Ochoa at NYU. When we

worked together, he did beautiful work—showing that you get the same type of phenomena with an adrenergic type 2 receptor as you get with the opiate receptor. He showed that antagonists of the adrenergic receptor result in the same kind of tolerance and increase in adenylate cyclase activity. He found that antagonists antagonize (reduce) the activity of adenylate cyclase or are inhibitors of the catecholamine norepinephrine-mediated activation of these receptors. It was the same kind of tolerance and dependence phenomenon that was seen with adrenergic receptors. We also did the same thing with acetylcholine receptors.

We were interested in the process of gene regulation there and the effect of cyclic-AMP in activating gene expression. We did a lot of work with the neuroblastoma cells for many years.

We cloned these cells. By cloning the cells, you start with a single cell, and all the cells in a line are derived from a single cell, single cell cloning.

So you have a relatively genetically homogeneous, population of cells derived from a single cell. They are all descendants of a single cell. If you have different clones, different clones have different properties. The tumor is heterogeneous. It consists, basically, of many different cell types. All of this work on our clones actually was derived from the mouse C1300 transplantable tumor. This was a spontaneous neuroblastoma that appeared in a mouse and was transplanted from one mouse to another. It was kept going by tumor transplantation for many years.

Gordon Sato independently cloned cells by using the same C1300 mouse neuroblastoma and established a number of clones from this tumor.[4] We established many clones. In fact, we found that this tumor contained some clones that synthesize catecholamines — they are adrenergic. They have dopamine, and they have tyrosine hydroxylase, so they make catecholamines. There are classes of neurons that make catecholamines that use either norepinephrine or dopamine as a transmitter whereas other clones that we established from the same tumor synthesized acetylcholine. This was the first time that neuroblastomas had been found that synthesized acetylcholine. After we published this, it was found that some human neuroblastomas—neuroblastomas are tumors of children by and large—synthesize acetylcholine as well. So we had both cholinergic and adrenergic clones of neuroblastoma that we isolated from the tumor.

.Perola (Nirenberg) was an expert on catecholamines and enzymes of metabolism. We began to work together after Sid Udenfriend left the NIH to become director of the Roche Institute of Molecular Biology in New Jersey. She had worked on catecholamines with Sid Udenfriend for many years and she published many papers on catecholamines. We found some adrenergic cell lines that synthesized catecholamines. She characterized the adrenergic cell lines and characterized the catecholamines that were being synthesized as well as the enzymes involved. That was some of the work that she did with neuroblastoma cells.

Harahiro Higashida, a superb electrophysiologist who came from Japan, did beautiful work on the cultured neuroblastoma cells forming synapses. He has become a leader in the field using neuroblastoma cells as model systems for all kinds of electrophysiology. He then learned molecular biology and characterized many kinds of muscarinic acetylcholine receptors. He has done beautiful work with the cells in Japan and is now a professor of neurobiology in Kanazawa. When I visited him in Japan, he spent six months learning how to perform the tea ceremony for my visit, and it was really wonderful. It is a very elaborate ceremony. He came in magnificent robes. He looked marvelous. He has just the features. He looked like a warrior in those robes, and his teacher, who had taught him the tea ceremony, was there. It was an extremely interesting occasion.

[William] Bill Klein did superb studies on the characterization of muscarinic acetylcholine receptors in some of the clonal lines of neuroblastoma cells that we had established. We worked out a binding assay that allowed us to count receptors using a radioactive quinuclidinyl benzilate, QNB for short. This was before it was commercially available. I contacted the organic chemist who originally had synthesized this compound, which is an antagonist of a muscarinic acetylcholine antagonist. It inhibits the action of the receptor and has a very high affinity for the receptor. He sent us some of the compound, QNB that he had synthesized. We then converted it to a radioactive form so that we could use it, incubate it, with the cells and then wash the cells afterwards and determine the number of specific binding sites for QNB. Later on it became commercially available and everybody used QNB as a ligand for these receptors. By the by, Klein and I developed a close rapport with some of these people. Bill Klein is now a

professor of neurobiology at Northwestern University. His first doctoral student, Hemin Chin, came here to the lab so I feel like a grandfather, in a sense. Hemin Chin is an independent investigator at the National Eye Institute here at the NIH.

RH: With respect to the work that you did on neuroblastoma, how did the research you did affect the field?

MN: We had a major effect on the field because these cell lines are used by people all over the world. I always thought that even if I didn't solve these problems, some of the young postdoctoral fellows, who were working with me, would do this. Every year we send cell lines to 100, 200, and 300 people, all over the world. People have used them for all kinds of studies on neurobiology, and they are still using them. We established many hybrid cell lines, many neuroblastoma cell lines, and many neuroblastoma hybrid cell lines.

After Lloyd Greene, one of the postdoctoral fellows, left the lab, he continued to do the same thing, to establish some cell lines. One of the cell lines that he established has become an excellent system and another widely used model system for studying cells from the nervous system.[5]

There were two limitations to the cell systems that we established, I think. The first was that the cells were polyploid, that is, they had more than the normal number of

chromosomes. One of the cell lines that Lloyd Greene established was diploid. It had the same number of chromosomes as normal cells. That was a big advantage.

When we first cloned the mouse neuroblastoma cells, we cloned them in the absence of nerve growth factor. Nerve growth factor is a hormone that is required for the survival of a certain class of sympathetic neurons. We selected neuroblastoma cells that did not require nerve growth factor for survival, and that was a mistake. Lloyd Greene assayed some of the cell lines and found that they actually had receptors for nerve growth factor. They synthesized the receptors, but the receptors were non-functional. So we repeated the work. We started again from the C1300 uncloned tumor to reclone in the presence of nerve growth factor and had a whole set of clones that were cloned in the presence of nerve growth factor.

Unfortunately, over Christmas vacation, the person who was taking care of all of the clones lost the entire set. It was an absolute disaster. We had put a lot of work into it, and she lost everything. We never went back to it. That was too bad because the cell line that Lloyd Greene cloned had functional nerve growth factor receptors, and that was a big advantage. Those cells have been used to explore mechanism of action of nerve growth factor.

But, in retrospect, it turned out okay. Those cell lines that we cloned are widely used, and every year, as I said, we send out cell lines to at least 150 to 200 investigators all over the world. I have people who have sent all of the lines to various countries. For some

countries, instead of our sending them out, I tell them, “You can get them from somebody who is closer to you.” They are widely used as model systems for all kinds of work—pharmacology, electrophysiology, biochemistry, and molecular biology.

RH: Can you give me just a ball park estimate of how many cell lines you developed?

MN: We have freezers full. We have the largest collection of cell lines from the nervous system of anybody in the world, I think. We have, quite literally, thousands of cell lines.

We have a computer index. Norma Heaton finally ***created a computerized data index file*** of our handwritten inventory of notebooks of where the cells were stored in the freezer. The computer index is one line per freezing or per set of vials frozen, and this index is about 500 pages long. There are just thousands and thousands of ‘freezings.’ But, there are 27 cell lines that have been studied most carefully. We have to limit the studies. Of those 27 cell lines, we have found about five cell lines that form synapses with high efficiency, and the rest of the 22 cell lines have specific defects that we have characterized in one way or another in the process of synapse formation.

RH: Is the NG108 cell line the most important cell line that you have? How is it currently used?

MN: That is the most characterized cell line that forms synapses that we have. We send them out to all sorts of people. People use it for all kinds of things. That's a cell line that we selected because they have large cell bodies. They are good for electrophysiology, and they have lots of receptors of different kinds. This is the line we used to study the effect of morphine addiction on the cells and they form synapses. You can innervate every muscle cell on the plate. We have clonal lines of muscle cells that can be innervated as well.

Time-Lapse Photography of Cells

MN: Let me just go back a little and pick up some of the things that we did. We found cell lines that would extend neurites when they were treated with elevated cellular cyclic-AMP levels, and I was interested in the biology of the process just to see what the cells did. For years I took time-lapse motion pictures of the cells under various conditions—looking at the process of axon extension and retraction, just to get the feel of what the cells do.

This is at the level of the light microscope, but these are time-lapse pictures, so it takes a frame every so often. Then you can run a motion picture, and you speed up time tremendously this way. An ordinary experiment would go for 24 hours or more, and you could view the entire process in five or ten minutes speeded up many-fold.

That proved, I think, to be very valuable. I rediscovered, I suppose, observations that people had made many years ago, but it gave me a feel for what cells do in a normal nervous system when they extend neurites. We reported on the phenomenon and we found that the cells preferentially adhere to different substrata. You could write your name on the plate, and they would grow, just like your name. You could lead them wherever you want and make them grow wherever you want them by laying down a monolayer of molecules to which they preferentially adhered. We found that they retract processes very rapidly. I studied it quantitatively and got numbers out of it. It is amazing how much information there is in a picture.

Chick Embryo Retina

MN: In 1963, Roger Sperry, one of the major figures in neurobiology—really the grandfather of neurobiology—wrote an extraordinary, theoretical paper proposing a simple method of establishing unique molecular addresses for cells in a tissue such as retina.[6] He said that two kinds of gradients of molecules at right angles to one another that sweep across the tissue—would confer specific labels, addresses, upon all cells in the matrix of cells. This would be a simple molecular mechanism for providing all the cells with unique addresses. This was simply a theoretical paper. He was thinking about adhesion there.

But, while we were working with monoclonal antibodies, I thought of a simple way of testing this hypothesis directly. We would take a piece of tissue from dorsal retina, for

example, of the chick embryo retina, and another piece from ventral retina, the center of the retina, right, left, and so forth. We would use these tissues as antigens, make monoclonal antibodies, generate clonal cell lines that make different kinds of antibodies, and then test the specificity of the antibodies. We would look for an antibody that would recognize an antigen that was non-uniformly distributed in the retina, that was most abundant, for example, in dorsal retina and least abundant in ventral retina, or something like this, and look for a gradient distribution.

Lo and behold, we found just what we were looking for. We called it TOP for topographic, an antigen that was present in high abundance in dorsal retina and was distributed in a concentration gradient across the entire tissue so that the least amount was present in ventral retina.

This was the first protein that had ever been found that was distributed in a gradient across a tissue, and it confirmed the hypothesis that Sperry had made. We did a lot of work with this. We characterized it very carefully, and we purified the protein. We found that it was a membrane protein present in highest concentration in dorsal retina and lowest in ventral. We found that it formed a concentration gradient, at least a 35-fold concentration gradient, across the retina, and defined the dorsal-ventral axis of the retina.

When [Joseph] Joe Moskal was in the lab, he purified the protein and characterized it. After Moskal left the lab, we tried later to clone the protein but failed to do it using the monoclonal antibody. I wanted to find out what the amino acid sequence of the molecule

was because that may have given us a clue as to the function of the protein. It was present in chick embryo retina but not in mammalian retina, and the gradient was formed as the retina was formed. We could do developmental sequence, time sequence. We could dissociate the retina and culture the cells. We found that cells from dorsal retina remembered how much TOP antigen to synthesize in culture and that they did not need to interact with other cells in order to synthesize the correct amount. So you destroy the antigen when you trypsinize the cells, but then they resynthesize the antigen. If you cultured dorsal retina alone, it had very high levels, or if you cultured ventral retina, it had very low levels. If you mixed them, it would just be additive.

We found an unidentified mechanism that allowed the cells to permanently remember their position, and we had a marker for position in the retina. It was a cell surface molecule so I thought it most likely was involved in selective adhesiveness. It probably may well have functioned as a guide, a positional marker, that told other cells the relative position of the cells in the retina. [David] Dave Trisler, [Michael] Mike Schneider, and Joe Moskal also worked on TOP on the gradient of molecules in the retina in chick embryo retina.[7]

It is difficult to get the numbers out, to quantify, but we saw all kinds of interesting phenomena there with Zvi Vogel. Zvi Vogel was a postdoctoral fellow from the neurobiology department at the Weizmann Institute of Science. When he came to the lab, we started to study acetylcholine receptors on muscle cells. We also used cells from the embryonic nervous system, like chick embryo retina, which we found you could

dissociate into single cells and then re-associate. They would form as many synapses in culture as they would in the intact retina, and you'd see the same variety of synapses forming. It is a remarkable and extraordinary phenomenon. We did quite a bit of work with normal cells and with embryonic rat brain dissociated into single cells.

Robert Ruffolo was an absolute live wire. He got his degree in pharmacology. He was an expert, extremely knowledgeable about adrenergic receptors because he'd worked on them for his thesis problem. He knew the ligands for different species of receptors much more thoroughly than anybody else did. The pharmaceutical people knew him from the time he was a graduate student and had spotted him as somebody whom they must hire. Today he is vice president of a major pharmaceutical company. He went at first to Kalamazoo, Michigan, I think, to Upjohn. I think he then left Upjohn and went to another pharmaceutical company.

He and I did some work that I have always considered extremely interesting. We used chick embryo retina cells. First, we removed chick embryo retina from about eight-day-old embryos, and dissociated the cells to single cells and then cultured the cells. We found that they could form synapses. My estimate is that they form about as many synapses in culture, after dissociation, as they do in the intact retina. Enormous numbers of synapses. We could see the same in the electron microscope. We could see the same kinds of morphologically distinctive synapses that were formed in culture as formed normally in the retina.

We found that some of the retinal neurons synthesize acetylcholine. We added them to striated muscle cells, which normally never forms synapses with retinal neurons, at least to my knowledge. We found that we could innervate every muscle cell on the plate. Don Puro and some others were also involved in this. It was remarkable. I treated it like a problem in enzyme kinetics. We could do quantitative calculations with these things. They were incredibly active in releasing acetylcholine and forming synaptic connections with muscle cells.

After being in culture for a certain length of time, we found that gradually all of the incorrect mismatched retinal neuron-striated muscle cell synapses were broken. They disappeared. Concomitantly, with the disappearance of these synapses, we found that the retinal neurons sorted out from the muscle cells and formed islands consisting of balls of cells, consisting only of retinal cells, not muscle cells. In fact, we found that they detached from the muscle cells and that the affinity of these balls for other retinal cells was so strong, it just pulled them off of the muscle cells. That is how the synapses were broken.

The cells were pulled right into these islands. You would have a cell on one end synapsing a muscle cell, and we'd sprinkle them. We would put the dissociated cells randomly on top of a monolayer of muscle cells so that they would be randomly located on the plate. We published a number of papers on this. It is an extraordinary phenomenon.

The footnotes below will be placed in a separate digital file for linkage to this file.

[1] John D. Minna (1941-) earned an A.B. in 1963 at Stanford University and an M.D. in 1967 from the Stanford Medical School. After serving at the NIH in various positions, he became chief of the Navy Medical Oncology Branch of the National Cancer Institute in 1981.

[2] Xandra Owens Breakfield received an A.B. in 1965 from Wilson College and a Ph.D. in microbial genetics in 1991 from Georgetown University. She served as a postdoctoral fellow from 1971 to 1974 at the National Heart, Lung and Blood Institute and joined the Yale Medical School faculty in 1974.

[3] William A. Catterall (1946-) received a B.A. in 1968 from Brown University and a Ph.D. in 1972 from the Johns Hopkins University. He served as a postdoctoral fellow in the Laboratory of Biochemical Genetics of the NHLBI from 1972 to 1976 and as a staff scientist there from 1976 to 1977. He joined the faculty of the University of Washington, Seattle, in 1977.

[4] Gordon Hisashi Sato (1927-) received a B.A. from the University of Southern California in 1950, a Ph.D. from the California Institute of Technology in 1955, and a D.Sc. in 1987 from the State University of New York, Plattsburgh. Sato was an assistant professor and then a professor of biochemistry at Brandeis University from 1958 to 1969, a professor of biology at the University of San Diego from 1969 to 1983, and chief executive officer of the W. Alton Jones Cell Science Center in Lake Placid New York in 1983 until retiring that same year. He has served as editor-in-chief of *Vitro Cellular Developmental Biology*. Among the several articles Sato published on animal cell culture were "Growth of HeLa Cells in a Serum-free Hormone–Supplemented Medium," *Advances in Pathobiology*. 1977(6)227-32; and with J.P. Mather, "The Growth of Mouse Melanoma Cells in Hormone-Supplemented, Serum-Free Medium," *Experimental Cell Research*., April 1979, 120(1)191-200.162.

[5] Lloyd A. Greene (1944-) received an S.B. from the University of Chicago in 1965 and a Ph.D. in 1970 from the University of California, San Diego. In 1987 he was awarded the CNR Medal of Excellence for contributions to neuroscience research. In 2002 he was affiliated with the New York University Medical School's Department of Pharmacology.

[6] Roger W. Sperry (1913-1994) received a B.A. in 1935 and M.A. in 1937 from Oberlin College and a Ph.D. in zoology in 1941 from the University of Chicago. In 1981 he was awarded the Nobel Prize in Physiology or Medicine for his research in the functions of the left and right hemispheres of the brain.

[7] G. David Trisler, Ph.D., joined the staff of the Department of Neurology of the University of Maryland School of Medicine, Baltimore. Michael J. Schneider (1938-) earned a B.S. at the University of Michigan in 1960 and a Ph.D. in 1964 at the University of Chicago. After serving as a resident research associate at the National Academy of Sciences from 1965 to 1967, he joined the faculty of the University of Michigan. From 1991 to his retirement in 2002, he served as interim provost and vice chancellor for academic affairs. Joseph Moskal (1950-) received a B.S. in 1972 and a Ph.D. in 1977 from the University of Notre Dame. In 1995 he was a professor and director for brain tumor research in the Department of Neurosurgery at Northwestern University Medical School.