A personal view from a long-lasting collaborator on the research strategies of Marshall Nirenberg

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1. 1976 was full of excitement

In 1974, after reading Dr. Marshall Nirenberg's paper on glioma and neuroblastoma cells (Amano et al., 1972), I wrote him a letter stating that I wanted to work with him as a post-doctoral fellow. I had previously studied membrane properties and potassium ions in neurons and glia cells in the rabbit cortex (Higashida et al., 1974). He responded to me in a letter with one sentence stating, "send me recommendation letters". After sending three letters, I patiently waited for his response, but there was none. I then asked Toshiharu Nagatsu, Marshall's friend who worked in the same laboratory (that of Udenfriend) at the National Institutes of Health from 1962-1964 (Nagatsu et al., 1964), to ask Marshall about the possibility of me obtaining a postdoctoral position in his laboratory. Then I received a response. Later, I learned that he rarely wrote letters and he only trusted the references of a few people. Nagatsu was one of his trusted friends, and once I met Marshall in 1976, we collaborated together intermittently for 35 years until just before his death on January 15, 2010. I believe that we formed a very personal bond.

In 1976, I started my post-doctoral training in the laboratory of Dr. Marshall Nirenberg. The first day I met Dr. Nirenberg was Monday, May 4, 1976. I waited for him to come into the laboratory since that morning, but he did not arrive until noon, which was usual at that time. Marshall attended the Monday lunch seminar in the laboratory library, but I did not recognize him. After the seminar, I had a chance to talk to him. When I saw Marshall in his office, which contained many books, journals, experimental notebooks and published articles,

although 49 years old, I thought he still looked young. There were only tiny spaces left around his desk and a path to his chair. Important information on experimental results, such as the properties of clonal cells, in bound files made 10-15 years before, was located exactly within reaching distance. On the shelf, there were many half-filled Coca-Cola cans.

At that time, many fellows engaged in electrophysiological research, including myself. Marshall introduced me, but not the other fellows, to Drs. Philip Nelson and Bruce Ransom who is now professors of Neurology at the University of Washington in Seattle. I observed them for 2 weeks to learn how to use both hands to handle the manipulators used to penetrate cultured neuroblastoma x glioma hybrid NG108-15 cells with microelectrodes under a phase-contrast microscope. It was necessary to train my left hand to move the microscope stage and penetrate presynaptic cells with a microelectrode. Furthermore, my right hand was trained to focus on cells, including post-synaptic muscle cells. At that time, other post-doctoral fellows who performed electrophysiological analyses quickly mastered recording miniature endplate potentials from cultured myotubes using the complete electrophysiological setup. Within 6 months, they could obtain meaningful data. In contrast, I first disconnected the wires from my setup and reconnected them from a preamplifier to a main amplifier, a pen recorder and a tape recorder. It took me 5 months to master the use of the recording system on cultured cells, and Marshall patiently watched my slow progress.

I was first able to detect MEPPs in co-cultures of NG108-15 cells and myotubes in September of 1976, which confirmed results in his laboratory,

originally obtained in the Nelson laboratory, of Marshall's own young fellow completely outside this field. Marshall always required confirmation of published data by a newcomer or that of ongoing data by mutual confirmation by 2 fellows in different rooms at the same time.

The year of 1976 was exciting in the Marshall laboratory because he achieved one of his goals in his neurobiological studies (Table 1). Ten years prior, he had decided to use neuronal tumor cells as a tool to decipher memory processes in the brain. Cell-cell interaction (recognition) was succeeded by *in vitro* functional cholinergic synapse formation between neuroblastoma x glioma hybrid cells and two different types of post-synaptic cells: primary muscle cells, which was observed in January (Nelson et al., 1976), and G-8 clonal muscle cells, which was observed in May (Christian et al., 1976).

The laboratory was full of excitement and activity. Every postdoctoral fellow waited in front of Marshall's office to discuss their new data that they wanted to publish in the *Proceedings of the National Academy of Sciences*. Marshall and each fellow often stayed in the laboratory without sleep on the night before the *PNAS* deadline each month to complete the final draft of their paper. The laboratory technician would rush to the *PNAS* office in downtown Washington, DC, to submit the final version of the paper on the day of the deadline. Once, Marshall said to me that submitting a *PNAS* paper with a limited number of words and printed pages is like writing a "Ha-i-ku", a Japanese poem composed strictly of 17 (5+7+5) Japanese characters.

2. Initial screening of neuroblastoma cells as presynaptic elements

Marshall's aim was to screen presynaptic elements for synapse formation capacity using 21 clonal cells or hybrid cells selected from his huge stock collected at the beginning of experiments (Table 2). Steve Wilson measured choline acetyltransferase activity and acetylcholine (ACh) content, and I measured ACh release in functional synapses identified by the presence of miniature or evoked endplate potentials in co-cultured myotubes. We tested 3-5 new clones per month. However, we obtained negative data for 6 months. This project seemed to be misguided in everyone's eyes. However, on May 5, 1977, I measured thousands of synaptic responses for more than 30 min from a myotube cocultured with NBr-10A (N18TG-2 x Bufferlo rat liver cell) hybrid cells. I rushed to Marshall's room where he was talking with Klee, and both came to watch the synaptic activity. In the same week, Wilson obtained a huge release of ACh from NBr-10A cells in response to high potassium (Table 2).

In May 1977, I determined that MBr10A cells were better than NG108-15 cells with respect to synapse formation, 1 week before the birth of my son. I stopped working for 1 week to take care of my wife and children without telling Marshall and the other people in the laboratory. When Marshall wanted to discuss the positive data, he was uncomfortable and slightly angry about my absence. This was the only time he showed emotion to me.

In some ways, synapse formation paralleled the ability to synthesize the ACh. However, the parallel was incomplete. Cells with higher synthetic ability formed more synapses, but many other cells with choline acetyltransferase activity did not form synapses (Nirenberg et al, 1983a and b). It appeared that additional factor(s) controlling ACh release remained undiscovered.

A clue to the missing factors was obtained 20 years later by researchers in Gif, France. In 1995, when I examined the ACh release capacity of ACh-deficient clones overexpressing choline acetyltransferase, I observed that there were two types of cells: those that can form synapses (ACh release+) and those that cannot (ACh release-) (Zhong et al, 1995a and b). The representative clones of the former and latter are Neuro2A and N18-TG2 neuroblastoma cells, respectively. In 1996, a French group determined that a 16-kDa proteolipid, a mediatophore in *Tordedo* electric organs (Israel et al., 1987), mediates Ca^{2+}-dependent ACh release from N18-TG2 neuroblastoma cells in which choline acetyltransferase and a mammalian ortholog, the c-subunit of the V0 sector of vacuolar proton ATPase (ATP6V0C; Di Giovanni et al., 2010), are exogenously expressed (Falk-Vairant et al., 1993). They pointed out that the capacity for synapse formation in NG108-15 cells derives from C6BU-1 glioma cells because ACh was released after the glioma cells were soaked in ACh solution, and C6BU-1 cells possess endogenous ATP6V0C and can release ACh. Marshall would not be so happy if the difference in synapse formation capacity could be partly explained by a property derived from glioma cells with ATP6V0C and not from neuroblastoma cells. However, this system was recently utilized to show that dopamine can be released from N18 cells with ATP6V0C and from NG108-15 cells, and that ATP6V0C mediates dopamine release in the lesioned striatum in hemi-Parkinsonian model mice (Jin et al., 2012, in this issue), indicating that ATP6V0C is a potential gene therapy agent for Parkinson’s disease.

3. Finding the best combination of PGE1 and theophylline for cell differentiation

Marshall did much work to measure adenylyl cyclase activity and intracellular levels of cyclic AMP (cAMP) and cyclic GMP, which were the essential aspects of hormonal signal research at the time (Matsuzawa, 1975; Sharma et al., 1975) even after Gilman left for Charlottesville, Virginia, in early 1970 (Gilman and Nirenberg, 1971). Marshall reached his favorite working hypothesis for opiate tolerance and dependence by showing the time course of sensitization and desensitization of adenylyl cyclase upon exposure to opiates, resulting in increases and decreases in cAMP content (Sharma et al., 1975). He seemed to keep this model as a model of *memory or cellular memory of neurons*. In 2009, based on this hypothesis, he identified enhancers of long-term memory via their activation of the cAMP responsive element binding (CREB) protein signaling pathway using a high-throughput screening format (Xia et al., 2009 and 2011).

In 1976, synapse formation was induced by dibutyryl cAMP-mediated functional differentiation in culture medium. In March of 1978, Marshall wanted more efficient cell differentiation agents to elucidate cAMP-dependent cell differentiation.

In August of 1978, Marshall suddenly got sick with very severe pain from gallbladder stones. He was admitted to the suburban hospital near our building (36) in the northeast corner of the NIH campus. Marshall continued to work during the night. He usually carried two folders in his arms, full of the day's papers and documents, and he went back to his house at around 5 or 6 o'clock

in his convertible with his wife, Perola. He would read these papers until late and fall sleep in the early morning. He did not look so strong but rather pale. We were very anxious to know if he suffered from an associated gallbladder tumor, but that was not the case. Due to his life style, whenever he came to Japan, he would say that he could easily adapt to the jet lag because he had lived in the United States on Japanese time.

When we visited him in the hospital after his operation, he smiled when he heard the results regarding PGE1 and theophylline (Nirenberg et al., 1983a and b; Higashida, Wilson, Keninner and Nirenberg, unpublished data).

4. Screening peptide receptors in NG108-15 cells

In the fall of 1977, Marshall showed me a new idea of his and asked me to try receptor screening in NG108-15 cells using electrophysiology. This method was used to measure membrane potential changes in response to peptides, and it seemed to have advantages over the biochemical assay with each radio-labeled ligand because the electric responses could be obtained in real time 1-2 min after the application of current to cells.

In 1977, A.V. Schally, R. Guillemin, and R.S. Yalow shared the Nobel Prize for identifying and assaying new hormones extracted mammalian tissues. At that time, it was beginning that peptide hormones were synthesized by a Beckman's peptide synthesis apparatus, and then synthetic hormones had become commercially available. Marshall was attracted to this convenient way of screening for peptide receptors, which were not known in neuroblastoma cells.

He made a list of more than 30 peptides to be tested using Sigma

catalog-type experiments. Most of the agents showed no response, with the exception of neurotensin, angiotensin II and bradykinin. Although bradykinin was not included in Marshall's original list, I added it because I was familiar with this pain-inducing peptide in my graduate work in Nagoya, Japan. We concluded that vasoactive peptides induce transient hyperpolarization followed by prolonged depolarization (Higashida et al., 1986), and no electrical responses were elicited by peptides that preferentially couple to adenylyl cyclase. However, at that time we did not know the mechanism by which these three agents produce such a complex pattern of membrane potential changes. I speculated that such complex sequential changes could be explained by cAMP, but by a different mechanism.

5. Novel signal transduction via phosphoinositide turnover

Six years later, in 1983, the above question was answered by my own effort with Yano and Nozawa from the Gifu University School of Medicine from which I graduated. Nozawa, a lipid biochemist, was interested in muscarinic ACh receptors (mAChRs), which were expected to couple to phospholipase C because Hokin in Canada (1953) and Michell (1975) and Berridge (2009) in the UK found diacylglycerol and inosito-1,4,5-trisphosphate as possible second messengers. Because I had neuroblastoma cells in culture in Japan, after completing my post-doctoral training in 2.5 years, we instantaneously performed experiments on lipid turnover in neuronal cells. Even though NG108-15 and N1E-115 neuroblastoma cells possess mAChRs, as shown in the dramatic elevation of cyclic GMP by carbamylcholine (Matsuzawa and Nirenberg, 1975), we struggled without results for 2-3 months. We then examined bradykinin

because I knew that these cells showed electrophysiological responses to this agent. The PI turn over was clearly elevated by bradykinin, and we published this result in the Journal of Biological Chemistry (Yano et al., 1984). This was the 5th example of receptor-mediated elevation of PI turnover, and of course the first report of this elevation by bradykinin (Berridge, 1986).

We later determined why ACh had no effect on PI turnover. When genes for receptors were found and cloned in the 1980s, each receptor consisted of several subtypes, revealing that NG108-15 hybrid cells and other clonal cells express only one receptor subtype, which was a surprise to all of us. NG108-15 and N1E-115 cells possess the m4 subtype of mAChRs (Fukuda et al., 1988), a finding confirmed by me, Fukuda, and Numa at Kyoto University, who obtained numerous genes encoding membrane elements in neurons, such as nicotinic and muscarinic acetylcholine receptors, voltage-dependent sodium and calcium channels, ryanodine receptors and GTP binding proteins (Numa et al., 1988; Numa, 1987-1988).

Based on our signal transduction studies, the m1 and m3 subtypes couple to phospholipase C and PI turnover and result in membrane potential changes. However, the m2 and m4 subtypes are selectively coupled to adenylyl cyclase. Therefore, these subtypes do not result in the activation of phospholipase and do not cause membrane changes (Fukuda, 1988). Consistent with this, in the initial experiment on NG108-15 cells, we observed no response with ACh.

In addition to synapse formation capacity, NG108-15 cells showed strong intracellular signal responses via intrinsic receptors. For a long time,

NG108-15 cells and other clonal cells were used to analyze the signals from intrinsic receptor subtypes. However, starting in 1988, I used NG108-15 cells to overexpress or transform extrinsic genes after transfection (a heterologous expression system). At that time, Marshall stopped using NG108-15 cells, although such new applications interested Marshall. He carefully stored his cells and answered requests for them for different purposes from all over the world.

6. Revolutionary idea of cloning neuronal tumor cells and hybrid cells

Marshall Nirenberg introduced a method for cloning cells commonly used in bacteriology to the study of mammalian neurons after decoding genes. Studying mammalian neuronal cells with a uniform genetic background was not seriously considered before Marshall's project, and this approach enabled us to characterize biochemical and pharmacological properties and the regulation of neurotransmitters, receptors, ion channels, and intracellular signal transduction. Thus, Marshall helped to found the new field of neurobiology. This cloning technique has been popular since its inception (Amano et al., 1972).

Starting in 1970, Marshall showed much interest in somatic cell hybrids as mentioned above (Table 1). I think Marshall thought he could dissect several neuronal properties in the developmental process. He produced hybrid cells to dissect their neuronal properties via a collaboration with H. G. Coon at the National Cancer Institute. John Minna, who was in Marshall's laboratory until 1976, and Marshall used hybrids between neuroblastoma and fibroblast cells (Minna et al., 1971) and proposed the following differentiation stages from primitive to mature neurons: neurite extrusion (morphological aspect), type B

currents (K channel properties), type A currents (sodium channels) and cholinergic properties with choline acetyltransferase. The expression sequence in development can tell us which neuronal properties are the most critical (Minna et al., 1972). Unfortunately, additional information such as the chromosomal location of these properties was not obtained because most hybrid cells possess tetraploid chromosomes that are a mixture of mice and rats (Kano-Tanaka et al., 1982; Higashida et al., 1985).

7. Dopaminergic clone

Another disadvantage of Marshall's clonal cells was that he failed to obtain true dopaminergic cells. He selected adrenergic clones due to tyrosine hydroxylase activity (Amano et al., 1972) because he belonged to one section of the Udenfriend laboratory. Udenfriend, Nagatsu and Perola Zaltmann-Nirenberg (who came from Brazil and spoke Portuguese) characterized tyrosine monooxygenase (hydroxylase) as the first step in tyrosine metabolism to adrenaline (Nagatsu et al., 1964; Udenfriend et al., 1965). As a result, tyrosine hydroxylase was very familiar to Marshall and Perola. Later, it became clear that these clones lacked the necessary enzymes to make dopamine, noradrenaline and adrenaline. Therefore, these clones were imperfect adrenergic cells. However, a more suitable PC12 pheochromocytoma clone with dopamine release was introduced by Lloyd Greene, an alumnus of Marshall's laboratory (Greene and Tischler, 1976).

In 2000-2001, when the big question of how to use stem cells in medicine arose, Marshall said, "the basic idea of using dopaminergic clonal cells

in transplantation therapy for Parkinson's disease has already been shown 30-40 years ago in my research".

7. Cell-cell recognition by monoclonal antibodies

In 1976, cell-cell interactions were the topic in Marshall's laboratory (Table 1), because two different cells form a synaptic connection (Nelson et al., 1976). Usually, heterophilic recognition (e.g., in liver and brain cells) is inhibited to promote aggregation. Synapse formation is a typical means of cell recognition. Sperry's hypothesis (1963) of the chemical gradient was interesting even to Marshall (Goolsby et al., 2012, in this issue).

In 1975, when Milstein published his technique for making hydridoma cells, which were made by fusing proliferating myeloma cells with antibody producing cells (Kohler and Milstein, 1975). Frank Walsh arrived in Marshall's laboratory in 1977 from London and brought with him an understanding of the new monoclonal antibody technology having heard the Milstein's lecture there. Marshall immediately saw the importance and potential of the method to identify cell-cell recognition molecules and this started a major new research area for the laboratory for a number of years, 7 years prior Milstein being awarded the Nobel price in 1984. His idea was to obtain monoclonal antibodies against neuronal surface molecules such as calcium channels and neurotransmitter receptors. These antibodies became useful for characterization of membrane proteins and cloning genes encoding. Therefore, Marshall immunized mice with neuroblastoma whole cell surface membranes as antigen instead of purified protein. Many hybridoma cells were established in this way but have remained

uncharacterized.

In 1980, Marshall was invited to the 23rd annual meeting of the Japanese Society for Neurochemistry, and when he came he asked me to come back and work with him on screening monoclonal antibodies for the following functional responses in dynamic synapses in culture: an increase or decrease in MEPP frequency. Within the 3 months from December 1980 to February 1981, I screened >20 monoclonal antibodies and classified them into 3 groups: those that increase or decrease MEPP frequency and ineffective antibodies. I presumed he wanted to find an antibody for voltage-dependent Ca^{2+} channels. Although we examined them extensively, most of the monoclonal antibodies raised from neuroblastoma cell surface membrane antigens turned out to recognize sugar moieties (glycoproteins) rather than a specific protein. Thus, all the results on monoclonal antibodies against neuroblastoma cells have remained unpublished. This was a sharp contrast from neural retina neurons, in which many interesting antibodies were reported (Eisenbarth et al., 1979; Trisler et al., 1981; Nirenberg et al., 1983a and b).

9. Bradykinin receptor signaling

When I recall being invited for the third time to work in Marshall's laboratory from 1984 to 1987, I always keep the following story in mind: Echu, the emperor's teacher and a Buddhist priest in China, called his attendant three times. The attendant responded three times. Echu said, "I thought I was the World-Honored One, now I realize that you too are the World-Honored One". Of course, I thought Echu was Marshall and that I was the attendant.

This time Marshall and I agreed to work on signal transduction downstream of bradykinin receptors. One day he introduced me to a resident scholar at the NIH, David A. Brown from University College London. We worked on the electrophysiology of cultured cells. Brown and I obtained enough data to show that bradykinin-receptor-induced membrane excitation occurs due to the closure of voltage-dependent time-independent potassium M-currents (Higashida and Brown, 1986). Thereafter, we worked together on the mechanism of IP3 and protein kinase C, and we proposed that these secondary messengers are responsible for biphasic membrane currents (Brown and Higashida 1988). Later, this hypothesis was applied to mACh receptors (Fukuda et al., 1988).

Using pertussis toxin, I, Klee and Nirenberg suggested that bradykinin receptors use the Gi or Go GTP binding proteins to couple to phospholipase C in signal transduction (Higashida et al., 1986). Unfortunately, this suggestion later turned out to be not completely correct (Taussing et al., 1992), although bradykinin receptors were determined to couple to pertussis toxin-sensitive G proteins. Pertussis toxin-insensitive Gq/11 G proteins were not known at that time, which could be one reason that this suggestion may have been incorrect (Smrcka et el., 1991; Taylor et al., 1991). However, our work stimulated a field of research on the links between signaling molecules and membrane currents.

10. The best image of a real scientist

Around 1985, Marshall tried to clone genes for Gαs proteins and Gαi G proteins (Bray et al., 1986 and 1987). He read amino acid sequences by

translating nucleotide sequences using a genetic code table. I frequently saw him through the open door of his room trying to do this. At that time, every researcher manually translated each triplet to an amino acid using this table. Every researcher in the molecular and biochemical fields owes him for this discovery, and he also used his code table in his gene cloning research. I thought to myself, “This is science!”

At that time, Marshall tried to clone bradykinin receptors. As a first step, he proposed to purify bradykinin receptors using [S^{35}]GTP-γ-S as a probe. He thought bradykinin receptors expressed in NG108-15 cells could be pulled down as radiolabeled GTP, G protein and bradykinin receptor complexes that could be trapped in filters (Figure 1), which is one of his favorite methods. Then, we could make a bradykinin receptor antibody or determine a partial bradykinin receptor amino acid sequence for gene cloning. He proposed that [Ca^{45}] release from HEK cells expressing bradykinin receptors from the transfection of candidate cDNAs could be used for characterization in multi-well plates. Unfortunately, the postdoctoral fellow from Japan who used this functional gene cloning method was not successful. However, a similar functional cloning strategy was successfully applied to capsaicin–receptor ion channels (Caterina et al., 1997).

11. Genome-wide screening for synapse-forming genes in fruit flies

The last project I worked on with Marshall was genome-wide screening for genes related to heart and neuron development using RNA interference (Ivanov et al., 2004; Koizumi et al., 2007). Marshall applied for a special grant from the National Heart, Lung, and Blood Institute and received money to initiate

genome-wide research in *Drosophila* embryos 2 years later than the discovery of RNAi by Fire and Mello (Fire et al., 1998), who won the Nobel Price in 2006. When I joined this project in June 2001, double-stranded RNA solutions were already in his hand. Three different solutions were mixed for injection into fly embryos to facilitate the screening. However, we later switched to testing each RNA solution in 100-200 fly embryos. This was my first time studying the *Drosophila* genome. Marshall taught me himself, and together we studied the neural structures in embryos. At first, I selected too many genes that destroy the formation of the central and peripheral nervous systems.

On September 11, 2001, terrorists attacked the World Trade Center in New York and the Pentagon in Washington, DC. At the end of September, Perola died after suffering from Alzheimer's disease. Approximately 10 years previously, her disease was first recognized by an impairment in her spoken English, her second language. She was cared for in their home by a nurse during the day and by Marshall at night. There was a funeral for her, but unfortunately I could not attend because I had to go back to Japan on a scheduled flight. And if I missed this flight, it would have been very difficult to arrange another flight. After the terrorist attack, there was a lot of confusion everywhere, especially on airplane flights. I briefly expressed my sincere condolences about her death to Marshall and left for Japan. In Japan, I slowly built up my fly research unit, continuing the project by myself with shared RNA solutions.

Before screening >5800 genes, I visited Marshall once or twice a year. During this period, he bought a new house in Potomac, Maryland, and he was

kind enough to let me stay in his house every time, which was a surprise to me at first. One reason he allowed me to stay was because of his marriage to Myrna Weissman, a professor at Columbia University who lives in New York. She is very energetic and open-minded . One time in 2002, Marshall told me about his idea of getting married again and asked me about the efficiency of my life style, living in two cities. After discussed with my wife, I remember answering that if you feel you are happy, then it is okay and there is no problem at all living separately during the weekdays and together on the weekend. I lived this way for more than 30 years with very little trouble. I believe that Marshall gained a vivid life surrounded by in-law family members, similar to how he lived in his younger days in Florida.

He also moved to his old location on the 7th floor in corridor D of Building 10, the main NIH building. I heard that he had moved to the same location in which he had conducted research on the genetic code. He used half of a room for his own office at his request. He didn't feel he needed all the space he had to do his work and didn't want to see it wasted when other scientists might be able to use it. He was quite content to have a small office. His new laboratory was downsized to one-third the size of his old laboratory in Building 36, where he used two floors (received after being awarded the Nobel price) and kept all kinds of supplies and constantly refilled chemicals. The liquid nitrogen for keeping culture cells was maintained by two technicians, and 4-5 secretaries were always in his office.

12. New studies on social memory and autism

In 2003, I published an article on the mechanism by which Kv7.2 (M-type) potassium channels are closed by protein kinase C and the A-kinase anchoring protein in response to ACh (Hoshi et al., 2003). Based on this publication and suggestions for faculty development by Marshall and Myrna, I was awarded a sizable grant from the central government at the Kanazawa University Center of Excellence Program on Innovative Brain Science on Learning, Memory and Development. In this program, I focused on social cognition, memory and autism spectrum disorders. When I examined male behavior in CD38 knockout mice, males showed impaired social memory in the form of amnesia regarding mates from previous encounters (Jin et al., 2007; Higashida et al., 2010). Two single nucleotide polymorphisms in human CD38 were determined to be associated with autism spectrum disorder (Munesue et al., 2010).

Additionally, I further characterized two genes, *Robo* and *Pebble*, which were identified as strong candidates for involvement in synapse formation in *Drosophila* embryos by the RNAi whole-genome project (Ivanov et al., 2003; Koizumi et al., 2007). Expression of the axon guidance receptors ROBO1 and ROBO2 was significantly reduced in the lymphocytes of an autistic group (Anitha et al., 2008) and in the anterior cingulate cortex and primary motor cortex of postmortem brains of autistic humans (Suda et al., 2011). My daughter, who met Marshall frequently from 3 to 5 years of age at the NIH kindergarten, and I showed that the Rho guanine nucleotide exchange factor Ect2, an ortholog of *Drosophila Pebble*, negatively regulates neurite outgrowth in neuroblastoma × glioma hybrid NG108-15 cells, and that this protein is expressed in the

developing mouse pituitary gland (Islam et al., 2010; Tsuji et al., 2011). The seeds sown by Marshall for deciphering brain memory are still growing here with regard to social memory and autism, which is one of the major topics in the 2010s.

Now Marshall takes his everlasting sleep in Sharon Garden, a Jewish cemetery in Valhalla, Westchester County, New York. His grave reads, “Marshall Warren Nirenberg. 1927–2010. A titan of science. A man beloved. He deciphered the genetic code and showed that all living things are related. Nobel Laureate Medicine and Physiology, 1968.” Myrna and I visited his new grave on July 27, 2011. I prayed for him by flattening my hands in the Japanese style, and I placed a small stone on his stone grave in the Jewish style.

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Table 1 Transition of Nirenberg’s targets and interests in1970s of the post-genetic-code era.

Year	1967-	1970-	1973-	1976-
Method	Cloning	Somatic cell hybrid	Co-culture	Hybridoma
Targets	Genetically pure population	Differentiation	Synapse formation	Monoclonal antibody
Interests	Neuro-transmitter	Developmental stage	Cell-cell recognition	Chemical recognition

Table 2. Acetylcholine synthetic activity and synapse formation in myotubes[&]

Cell lines	Parental clones	Choline acetyltransferase (pM [^{14}C]ACh formed /min/mg protein)	Synapse formation (%)
NBr-10A	N18TG-2* x BRL-30E**	201	65
NG108-15	N18TG-2 x C6BU-1***	191	55
NBr20A	N18TG-2 x BRL-30E	188	40
NCB20	N18TG-2 x CHEC#	37	20
SB37A	N18TG-2 x NS-20TG11-E*	178	14
NS20Y	Neuroblastoma clone	245	6
NG108-05	N18TG-2 x C6BU-1	349	6
NS27B	Neuroblastoma clone	168	4
N4G-Ba	N4TG-3* x C6BU-1	386	3
N4G-Ca	N4TG-3 x C6BU-1	463	1
N4G-Cb	N4TG-3 x C6BU-1	182	0
SB37B	Neuroblastoma clone	152	0
SB53B	Neuroblastoma clone	72	0
SB76B	Neuroblastoma clone	61	0
SB21B	Neuroblastoma clone	34	0
SB50A	Neuroblastoma clone	22	0
SB79A	Neuroblastoma clone	22	0
SB501A	Neuroblastoma clone	22	n.d.
SB66B	Neuroblastoma clone	10	0
SB602A	Neuroblastoma clone	5	0
NBr9A	N18TG-2 x BRL-30E	5	0

[&] Taken from unpublished data of Wilson, Higashida, Adler and Nirenberg and Nirenberg et al., 1983a and b. *Neuroblastoma clones; ** Buffalo rat liver cells; ***rat glioma cells: #Chinese hamster embryonic brain cells.

Figure legends

Fig, 1. One of hand-writing protocols, showing his idea of purification of bradykinin receptors (BK and R). A bottom cartoon represents the filter trap method, one of Marshall's favorite methods, which were used in studies of protein synthesis from synthetic mRNAs and adenylyl cyclase activity. α,γ,β, subunits of GTP binding proteins; GTPγS, non-hydrolysable GTP; S^{35} Ca^{45}, isotopes of sulfide and calcium as tracers.

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