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TIMELINE

Ricardo Miledi and the calcium hypothesis of neurotransmitter release

Jade-Ming Jeng

Ricardo Miledi has made significant contributions to our basic understanding of how synapses work. Here I discuss aspects of Miledi's research that helped to establish the requirement of presynaptic calcium for neurotransmitter release, from his earliest scientific studies to his classic experiments in the squid giant synapse.

"The arrival of an action potential at an axon terminal causes a rise in the cytosolic Ca^{2+} concentration, which triggers exocytosis of the synaptic vesicles and release of transmitter." — *Molecular Cell Biology*[1]

"Experiments at the squid giant synapse, where the presynaptic nerve terminal is large enough to permit the insertion of microelectrodes, show that an increase in intracellular Ca^{2+} in the absence of depolarization stimulates transmitter release. Thus, Ca^{2+} is both necessary and sufficient for secretion." — *An Introduction to Molecular Neurobiology*[2]

The above excerpts, both taken from current introductory, college-level textbooks, describe a concept that is universally accepted as a basic and fundamental principle in neuroscience: that Ca^{2+} is required at an axon terminal for vesicular neurotransmitter release to occur. Although it can be difficult (and most often an oversimplification) to attribute such advances in scientific knowledge to a single person, Hall[2] highlights the importance of a set of experiments that were carried out in the squid. And in this case, the key experiments, reported in 1973 in the *Proceedings of the Royal Society of London* in a paper entitled "Transmitter release induced by injection of calcium ions into nerve terminals"[3], were indeed carried out and reported by one author — Ricardo Miledi, at present Distinguished Professor in the Department of Neurobiology and Behavior at the University of California, Irvine, and Investigador Titular at the Centro de Neurobiología, Universidad Nacional Autónoma de México.

Miledi's name is most frequently associated with his long-time collaborator and mentor Bernard Katz for their analysis of synaptic 'noise' at the neuromuscular junction (NMJ), but his singular work on presynaptic Ca^{2+} in the squid constitutes an equally significant milestone in our understanding of the synapse. Here I consider Miledi's career in the context of his exploration of 'the calcium story', and give an account of the succession of Miledi's experiments that established the calcium hypothesis of neurotransmission, which he and Katz put forward in 1967 simply as this: depolarization → calcium influx → quantal transmitter release[4]. I discuss Miledi's definitive experiments in the squid, which established the presynaptic requirement for Ca^{2+}, and consider them in view of the intellectual and technical developments of the time. How did Miledi develop his ideas about calcium, and what led him to pursue the avenues of investigation that resulted in the seminal 1973 paper?

The beginnings

In reviewing the literature leading up to Miledi's 1973 paper, there is a relative absence of directly competing or dissenting work, perhaps indicating that few investigators were curious at the time about the role of Ca^{2+} at the synapse. To place Miledi's curiosity in context, it is necessary to consider what was known then about the physiological importance of Ca^{2+}.

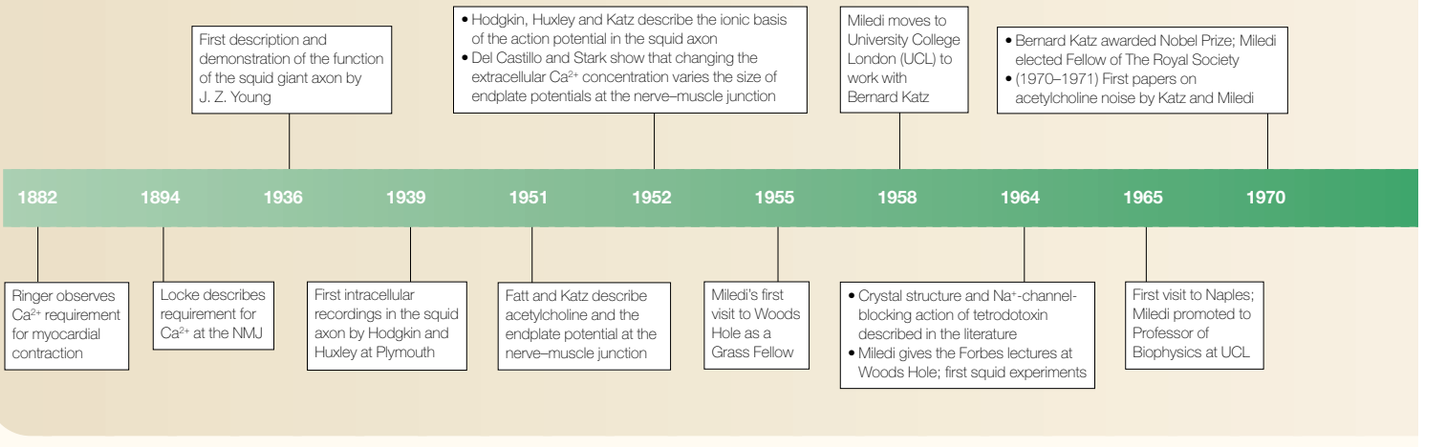
One of the earliest indications that Ca^{2+} was a vital ion in human physiology came from the work of Sydney Ringer, the nineteenth-century British physician and physiologist who developed the formula for a physiological saline, subsequently named after him, which has become the standard in experimental studies of tissues and organs, and clinical intravascular volume expansion in humans. The first description of Ringer's solution appeared in 1882 (TIMELINE), in a paper detailing experiments that he undertook to "ascertain the influence each constituent of the blood exercises on the contraction of the [frog heart] ventricle."[5] However, in an article published shortly afterwards, Ringer revealed that he had not been able to build on his earlier results because "I discovered that the saline solution which I had used had not been prepared with distilled water, but with pipe water supplied by the New River Water Company [in London]."[6] Ringer's thorough analysis of that pipe water revealed that it contained a significant amount of calcium, providing the first evidence that Ca^{2+} was required for muscle contraction in the heart; the German scientist Locke[7] extended this observation to the NMJ in 1894.

Miledi was familiar with Ringer's findings as a result of his medical studies at the Universidad Nacional Autónoma de México. As a young medical student in Mexico City in the early 1950s, Miledi was required to carry out 'social service' as part of his training, either by serving clinically in a medically understaffed part of the country, or by conducting research at a federally funded institute. Although he was described as a good physician by those around him, Miledi himself was convinced that he would make a terrible clinician; he imagined that he would end up seeing only one patient per week, because he would always be too interested in every unknown detail of the case, trying to work out how medicines might act. In fact, Miledi reveals, "I would not have finished medical school at all, except [Mela, his girlfriend, now his wife] refused to marry me unless I had earned my degree!"[8] Accordingly, when the time came for his social service, he chose to pursue a research fellowship at the Instituto Nacional de Cardiología with the esteemed physiologist Arturo Rosenblueth. In the course of his studies on the electrical origins of ventricular fibrillation, Miledi was able to nurture his scientific curiosity through a voracious amount of reading, teaching himself how to dissect out single nerve fibres and pull glass micropipettes by hand.

Figure 1 | **Woods Hole, 1955.** Ricardo Miledi (left) and Albert Grass.

In 1954, Albert Grass (FIG. 1) and Stephen Kuffler visited the Institute, looking for young investigators for the nascent Grass Fellowship

Timeline | **Calcium and neurophysiology: Ricardo Miledi's contributions**



Program at the Marine Biological Laboratory in Woods Hole, Massachusetts. Miledi demonstrated his techniques of microdissection and micropipette fabrication, in which he pre-filled the glass pipettes with salt solution before pulling their tips. Grass and Kuffler thought that his method was quite ingenious, and invited him to become one of the first Grass Fellows the following summer. Jumping at the chance to immerse himself in such a scientific environment, and to travel outside Mexico for the first time, Ricardo Miledi ventured north in the summer of 1955 to study lobster stretch receptors, and garnered along the way his first introduction to the giant synapse of the common squid, *Loligo pealii*.

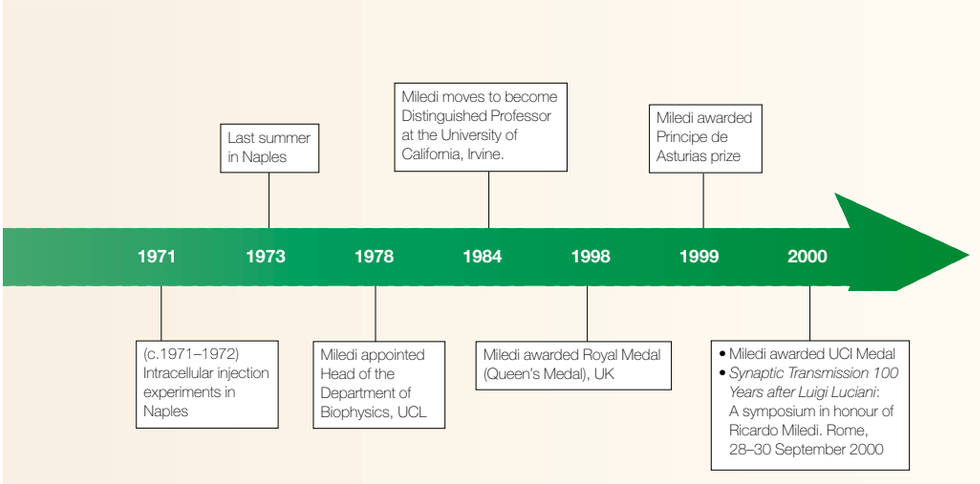
The summer in Woods Hole also served to remind Miledi of the importance of Ca^{2+} in synaptic transmission; the artificial seawater used in squid preparations contained a relatively high concentration of Ca^{2+}, and on more than one occasion, neglecting to add enough Ca^{2+} to his solutions caused his experiments to go awry. Furthermore, a paper that had been published recently by Del Castillo and Stark[9] caught Miledi's attention. It showed that changing the concentration of Ca^{2+} in the extracellular media bathing a frog neuromuscular preparation could lead to variations in the size of the endplate potential, and that removing all Ca^{2+} caused the potential to disappear. Clearly, less Ca^{2+} resulted in less transmitter release, but the question was why? Was Ca^{2+} flux across the membrane required to allow the propagation of action potentials along the unmyelinated nerve terminal branches, analogous to the roles of Na^+ and K^+, or were Ca^{2+} ions part of some intracellular molecular process, as in muscle contraction, which could somehow lead to the quantal release of transmitter?[10] Clouding the issue was the fact that it was generally believed at the time that the nerve impulse did not invade the nerve terminal. With these issues and his experience at Woods Hole fresh in his mind, Miledi returned to Mexico to finish his fellowship and begin to look for further research opportunities. Interestingly, the paper by Del Castillo and Stark was published in the very same issue of *The Journal of Physiology* as the landmark papers of Hodgkin, Huxley and Katz that described the action potential in the squid giant axon. It seems plausible that most of the attention of physiologists at the time was focused on these findings, rather than on any of the other results reported in that issue.

London: Ca^{2+} at the NMJ

In 1958, during an 18-month fellowship with John Eccles in Canberra, Australia, Miledi met Bernard Katz, who had described, in 1951, the quantal release of acetylcholine (ACh) and miniature endplate potentials at the NMJ. Towards the end of his stay in Australia, Katz offered Miledi a position in the Department of Biophysics at University College London, the institution that had nurtured Sydney Ringer nearly a century before. On his arrival in London, Miledi worked on several projects using the frog nerve–muscle preparation, examining neuromuscular fatigue and ACh release, ACh receptor expression at denervated muscle, and other related questions. It was not until the early 1960s that the theme of Ca^{2+} began to significantly reassert itself in his work. As Miledi recalls: "I had been mapping the distribution of the ACh receptors along the fibres of normal and denervated muscles by iontophoretic application of ACh. I then thought to do experiments where we would remove all the Ca^{2+}, and then apply the Ca^{2+} iontophoretically, to only a minute region of the muscle fibres, and that's more or less the way the whole [calcium story] began … Those experiments with Katz proved conclusively that in zero-Ca^{2+} medium, the nerve impulse still fully invades the nerve terminal, but does not release any neurotransmitter. And then as soon as you give a little Ca^{2+}, you get neurotransmitter release."[8]

In 1965, Miledi and Katz published their account of the effect of Ca^{2+} on ACh release from motor nerve terminals. They completely removed Ca^{2+} from the external environment of a frog neuromuscular preparation, then focally reapplied it to "locate the site of Ca^{2+} ion action in the transmission process."[11] A point of debate at the time was whether Ca^{2+} ions had an essential role in the mechanism (then unknown) by which depolarization of the nerve terminal increased the probability of quantal transmitter release, or, alternatively, whether Ca^{2+} concentration was a crucial element in the forward spread of depolarization along the axon to the nonmyelinated nerve terminals. In essence, this was also partly a debate as to whether the synapse was chemical or electrical in nature. The paper concluded that "the action of Ca^{2+} is concerned directly with the release of the transmitter, and not indirectly … by facilitating propagation or increasing the amplitude of the terminal nerve spike."[11]

Having shown that Ca^{2+} was required for transmitter release, and that the nerve impulse invaded the axon terminal, Miledi wanted to explore further the mechanism of Ca^{2+} action. He and Katz designed a set of experiments aimed at characterizing the synapse by describing its synaptic transfer, or input/output properties; that is, the relationship between presynaptic electrical input and the output of postsynaptic potentials caused by neurotransmitter release. However, to do so rigorously, Miledi needed to control the potential in the nerve terminal precisely, so that the detection of the postsynaptic potential would not have to rely on the all-or-nothing nature of the action potential in the presynaptic terminal. The technical hurdle was finding a way to accomplish that. The solution arrived in the form of the Japanese puffer fish poison tetrodotoxin (TTX), a selective blocker of Na^+ conductance, the usefulness of which had been described to Miledi and his colleagues by John Moore and Toshio Narahashi in 1964 (REF. 12).



A second and equally significant challenge was the small size of the presynaptic terminal at the NMJ, precluding the possibility of presynaptic electrode placement for current injection. It was then that Miledi thought: "maybe we needed to do some work on the squid, where I knew there was a giant synapse."[8]

Woods Hole revisited: August 1964

In the summer of 1964, Miledi returned to Woods Hole to give the Forbes Lectures, and seized the opportunity to work with the squid preparation during his visit. In the giant synapse, which is roughly 1 mm long, a microelectrode can be placed within the presynaptic axon in the synaptic region, and the effects of local polarization can be examined without complications introduced by changes in the propagation of the presynaptic nerve impulse, as would occur in the frog. Because there was some question as to whether the squid synapse was electrical or chemical, Miledi and his graduate student Clark Slater first repeated in the squid giant synapse the experiments that he had just done with Bernard Katz at the frog NMJ. To their delight, they discovered that the giant synapse behaved exactly as the NMJ did: presynaptic stimulation did not produce any detectable postsynaptic response in Ca^{2+}-free media, and focally applying extracellular Ca^{2+} restored synaptic transmission.

Miledi recalls that "because in the squid you can go inside the presynaptic terminal with an electrode, I then thought, well, I'll go into the nerve terminal and release Ca^{2+} inside there with zero Ca^{2+} outside, and if all I need is the Ca^{2+} inside, then the nerve impulse should release neurotransmitter…[But back then], when I injected Ca^{2+}, I didn't see [any postsynaptic response]."[8]

Puzzled by the outcome of this very first attempt at the Ca^{2+} injection experiment, Miledi considered how to interpret his results. As he had been working extensively with neurotransmitter receptors in London, he suggested the possibility of an extracellular receptor at the synaptic region, speculating that synaptic vesicles might dock at the presynaptic membrane, but then require the combination of extracellular Ca^{2+} and a Ca^{2+} receptor at that location to release their neurotransmitter cargo. But despite working for long hours while at Woods Hole, Miledi and Slater did not have enough time to carry out more experiments to test this hypothesis. Although the idea seemed to be a logical explanation of their data, Miledi still had his doubts; as he and Slater had not seen any miniature synaptic potentials at the giant synapse, there also remained the possibility that some methodological variable was occluding the ability to detect quantal release.

Equally frustrating was the fact that there was no time to attempt his experiments with TTX. Miledi had wanted to characterize the synapse by constructing a full input/output curve, but without TTX to block the nerve impulse, the presynaptic potential could be varied only up to a certain point, before the axon fired an action potential. In the paper published from Miledi and Slater's work at Woods Hole[13], they provided a partial input/output curve — measured by carefully changing the presynaptic potential to vary the amplitude of the action potential (a very difficult technical feat at the time) — but the obvious experiment was to repeat the measurements with TTX. With these ideas still waiting to be addressed, but his time in Woods Hole at an end, Miledi reluctantly went back to London.

Trawling in Plymouth

Miledi returned to University College London full of ideas for further squid experiments; despite his inability to detect transmitter release by postsynaptic potentials after the intracellular injection of Ca^{2+}, he felt that his own tentative suggestion of an extracellular Ca^{2+} receptor required further testing. But he now faced a significant practical obstacle: he was unable at that point to procure enough funding for a return to Woods Hole to continue his squid work. Meanwhile, by publishing the half-finished input/output curve, Miledi's ideas for his next set of experiments (recording from the squid in the presence of TTX) were easy for his colleagues and competitors to deduce and subsequently try as well.

One day, a disheartened Miledi encountered J. Z. Young, the man who had first described the usefulness of the squid giant axon in 1939, in the corridor at University College London. Miledi related to Young his dilemma about squid and being unable to return to Woods Hole. Young suggested that Miledi go to the marine station at Plymouth, where Hodgkin and Huxley had done all of their work on the giant axon.

Miledi followed this advice, but things did not go at all smoothly. On arriving in Plymouth, he discovered that the laboratory trawlers routinely brought back squid that were already dead. And although the squid axon retains its membrane properties even if the squid itself has been dead for a few hours, the giant synapse rapidly expired after the animal did.

Miledi tried in vain to resuscitate the synapses, adding glucose, amino acids and various other compounds to the preparation. He even attempted to dissect squid just after it was collected on the trawler, on a particular occasion when Bernard Katz was with him in Plymouth and both of them ventured into the rough seas. But despite all of his efforts, Miledi could not get a live synapse preparation, or revive one, at Plymouth. So, he returned once more to London, having learned mainly that "I'll never go fishing again for the rest of my life."[8]

Stazione Zoologica Napoli: 1965–1973

Back at University College London, Young encouraged Miledi not to give up, and this time directed him to the marine station in Naples, where investigators could procure live squid from an on-site aquarium. Katz, in turn, helped Miledi to secure travel funds from the Royal Society, which would allow him to go to Italy during the summer. At last, things had begun to fall into place.

Miledi was now in the midst of an incredibly productive time in his career. During the year, he worked in London with Katz, continuing to study ACh and beginning to develop their theories on noise analysis. Each summer, he spent from 2 to 8 weeks in Naples, doing as many experiments and collecting as much data as he possibly could while he was there. A consideration of all his work in the squid, including physiology, electron microscopy and nascent Ca^{2+} imaging, would be beyond the scope of this article. However, in his efforts to prove the calcium hypothesis, and with his conviction that he ought to be able to inject Ca^{2+} presynaptically and see the resulting neurotransmitter release, he overcame several key experimental obstacles, allowing him to succeed where his colleagues had not.

Synaptic suppression potential. On the first visit to Naples, Miledi and Katz immediately set to work measuring the full input/output curve of the giant synapse by blocking action potential firing with TTX[14]. At the same time, Rodolfo Llinás, Kiyoshi Kusano (FIG. 2) and their colleagues were carrying out similar experiments with squid at Woods Hole[15,16]. So, although the delays in securing a place to carry out the squid experiments had been wearisome, the time had also allowed interest in the calcium story to take root in the scientific community. Moreover, the delay had given Miledi the opportunity to consider further his results from Woods Hole, and plan an additional set of experiments.

It occurred to Miledi that perhaps there was not a receptor at the synapse, as he had

speculated in the paper with Slater, but a channel for Ca^{2+} instead. He recalls: "I was doing a lot of iontophoresis, so … [the concepts of] potentials and how ions could flow through a little hole … [were] in my mind a lot. I thought, if it's a channel, then we ought to be able to shift the potential so as to make the inside of the nerve terminal of the squid so positive that Ca^{2+} doesn't go in."[8]

But carrying out such an experiment required technical skill and more than a bit of patience. Because the Ca^{2+} concentration was so much higher in artificial seawater than inside the squid, a large electrochemical potential was required to suppress the putative inward Ca^{2+} flux during depolarization. Using TTX to block action potential firing was not enough, by itself, to permit depolarization of the presynaptic terminal to the positive potentials that were required to suppress Ca^{2+} influx. Ultimately, blocking K^+ by the intracellular iontophoresis of tertraethylammonium (TEA), in addition to TTX application, allowed Miledi to apply long depolarizing steps to the nerve terminal, eventually reaching a positive internal potential (the suppression potential) at which transmitter release was completely suppressed; when the depolarizing step ended and the potential began to fall, transmitter release was restored[17]. The findings indicated that once Ca^{2+} ions overcame the electrochemical potential necessary to enter the presynaptic terminal, neurotransmitter was released; this insight led to the first formal statement of the calcium hypothesis[4].

Intracellular injection of Ca^{2+}. Having provided a strong argument for the first half of the calcium hypothesis — that depolarization led to Ca^{2+} influx through a channel — Miledi turned his attention back to the idea of injecting Ca^{2+} into the presynaptic terminal to elicit transmitter release, reconsidering why his first attempt in Woods Hole had not yielded any postsynaptic response.

The key, Miledi reasoned, was to make certain that he could detect miniature postsynaptic events at the giant synapse, something that many, including himself and Slater, had previously attempted without success. Doing so would establish a less equivocal negative control for sensing changes in presynaptic transmitter release than he had had at Woods Hole. He decided to work with very small squid to reduce the dimensions of the synapse, as adequate space clamp was difficult to achieve at the time. As a result, he was able to see miniature synaptic potentials[18]. With the most problematic aspect of his first attempt at Woods Hole now resolved, Miledi could finally proceed with the Ca^{2+} injection experiment.

The injection experiments were carried out in the summers of 1971 and 1972, and published in 1973 in a straightforward paper[3]. The data were a classic illustration of the calcium hypothesis that Miledi had proposed with Katz in 1967: "inward movement of a positively charged Ca^{2+} compound, or of the calcium ion itself, constitutes one of the essential links in the 'electro-secretory' coupling process of the axon terminal."[4] By replacing external Ca^{2+} with Mn^{2+}, such that presynaptic depolarization produced no postsynaptic response, and restoring quantal postsynaptic potentials by injecting Ca^{2+} presynaptically, Miledi elegantly and efficiently showed the necessity and sufficiency of Ca^{2+} for transmitter release.

Figure 2 | **Stazione Zoologica, Napoli, 1973.** From left to right, Rainer Martin, Kiyoshi Kusano, Jacques Stinnakre and Ricardo Miledi.

The Ca^{2+} injection experiments would turn out to be a watershed in Miledi's career. In the early 1970s, the administration and organization of the Stazione Zoologica was in transition[19], making Miledi's continued collaboration with them increasingly more complicated[8]. In addition, Katz had been awarded the Nobel Prize in 1970, and with the added recognition came additional responsibilities outside the laboratory, leaving Miledi to assume a greater role in the university, and in teaching and training. Moreover, Katz and Miledi's work on noise analysis[20,21] was moving to the forefront, and Miledi's scientific focus was shifting from the presynaptic to the postsynaptic domain. All of these factors contributed to the eventual end of Miledi's era in Naples; he continued to publish work done with squid at the Stazione as late as 1986, but ceased his annual trips to Italy in 1973.

Impact of the papers

Miledi's contemporaries recall the reception of the squid papers by the scientific community: "These were very difficult experiments technically, so when others started seeing the work being done … the general consensus was an appreciation of Ricardo's technical finesse. The [scientific] story [itself was] so clearly put together that there was not much dispute about [the findings or the interpretation] at all" (I. Parker, personal communication).

Indeed, the response to the calcium papers, and the 1973 paper in particular, was not so much one of celebration or controversy as it was of productivity: Miledi's work on the calcium hypothesis, both singly in the squid and together with Katz at the frog NMJ, provoked a rapid succession of discoveries, including studies by Llinás and his colleagues both concurrent with[22] and subsequent to[23] the 1973 paper, further supporting and expanding the calcium hypothesis.

Moreover, the current study of molecular interactions between vesicular and plasma membrane proteins in exocytosis can trace its roots back to the calcium story and the crucial information that Miledi's work supplied, establishing Ca^{2+} as the lynchpin of the excitation–secretion coupling process. As with many historically significant events, it is only with the benefit of hindsight that the ground-breaking importance of Miledi's work, in terms of the way in which we understand and study synapses in general, can be fully appreciated.

Conclusions

I introduced this account of Ricardo Miledi's work on presynaptic Ca^{2+} with a pair of excerpts, highlighting the relevance of Miledi's experiments and conclusions from the squid. It is perhaps appropriate, then, to end with the following statement, underscoring the importance in neuroscience of the ion that so captured Miledi's curiosity and imagination that he travelled across continents — from Woods Hole, Massachusetts, to Plymouth, England, and finally to Naples, Italy — seeking to understand the role of Ca^{2+} at the synapse:

> "As a broad generalization, excitable cells translate their electricity into action by Ca^{2+} fluxes modulated by voltage-sensitive Ca^{2+} channels … Ca^{2+} channels … serve as the only link to transduce depolarization into all the nonelectrical activities controlled by excitation. Without [them] our nervous system would have no outputs."
> — *Ionic Channels of Excitable Membranes*[24]

Jade-Ming Jeng is a member of the Medical Scientist Training Program and the Department of Neurobiology and Behavior, University of California, Irvine 92697-4292, USA.
e-mail: jeng@uci.edu

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