Post-transcriptional modifications and “Calmodulation” of voltage-gated calcium channel function: Reflections by two collaborators of David T Yue

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This review article is written to specially pay tribute to David T. Yue who was an outstanding human being and an excellent scientist who exuded passion and creativity. He exemplified an inter-disciplinary scientist who was able to cross scientific boundaries effortlessly in order to provide amazing understanding on how calcium channels work. This article provides a glimpse of some of the research the authors have the privilege to collaborate with David and it attempts to provide the thinking behind some of the research done. In a wider context, we highlight that calcium channel function could be exquisitely modulated by interaction with a tethered calmodulin. Post-transcriptional modifications such as alternative splicing and RNA editing further influence the Ca\textsuperscript{2+}-CaM mediated processes such as calcium dependent inhibition and/or facilitation. Besides modifications of electrophysiological and pharmacological properties, protein interactions with the channels could also be influenced in a splice-variant dependent manner.

Reflections by Tuck Wah Soong, Singapore

Alternative splicing of P/Q-type Ca\textsubscript{V}2.1 Ca\textsuperscript{2+} channels in the brain

My entry into examining alternative splicing in calcium channels was initiated largely by the hypothesis that a certain splice variant of the Ca\textsubscript{V}2.1 gene codes for the P-type calcium channel that exhibits biophysical and pharmacological properties similar to native cerebellar P-type Ca\textsuperscript{2+} currents. During my postdoctoral years in Terry Snutch’s laboratory at the University of British Columbia, the calcium channel community was unable to recapitulate the native P-type calcium channel properties in heterologously expressed Ca\textsubscript{V}2.1 channels in either oocytes or HEK 293 cells. Mounting evidences however suggested the α-1A variants of the Ca\textsubscript{V}2.1 gene codes for the P- and Q-type calcium channel but the biophysical and pharmacological properties of the heterologously expressed α-1A cDNA in oocytes and native currents were different. One likely reason was that a splice variant of Ca\textsubscript{V}2.1 would possess the correct channel properties and another reason was that there was a requirement for an unknown factor or auxiliary subunit to associate to modulate the channels within the cellular milieu of the native neurons. Screening through and analyzing multiple Ca\textsubscript{V}2.1 cDNAs resulted in the identification of one almost full-length clone that differed from the original α-1A at 3 sites: an insertion of a valine in the I-II loop, and insertion of a dipeptide (\textit{-NP-}) in the domain IV S3-S4 linker region and 2 mutually exclusive exons that form part of the EF hand in the carboxyl region. By employing chimeric studies of natural variants of the α-1A, we were able to assign the altered electrophysiological and pharmacological properties to the inclusion or exclusion of the different alternatively spliced exons. For example, the inclusion of the \textit{valine} would influence PKC and G-protein modulations of the channel; inclusion of the \textit{-VNP-} would shift the IV curve to the more depolarised potential and decrease the sensitivity of the P/Q-type channel to inhibition by \textit{ω-AgaIVA}, a selective toxin blocker of the channel.\textsuperscript{1} While the functional characterizations of these splice isoforms brought us closer to a molecular structure of the P-type calcium channel that mimic native current, there were still uncertainties with regards to the presence of these isoforms in the cerebellar Purkinje cell, in which the P-type Ca\textsuperscript{2+} current was first described.

Besides Terry who has helped my career tremendously, another calcium channelologist who was also an inspiration to me was the late David T. Yue from the Johns Hopkins University School of Medicine. As this article is a tribute to David Yue, I will elaborate more about how I began and maintained my collaboration with him and his group and I do apologize that because of the intention of this review, I may have inadvertently left out many significant publications in the field.

In 1999, I obtained a Research Scientist Award from the Singapore National Medical Research Council to allow me to spend one year as a visiting scientist in the laboratory of William S. Agnew, chair of the Department of Physiology, the Johns
Hopkins University School of Medicine. While in Bill’s laboratory, I decided to use a method, developed by Scott Mittman and Mark Emerick, which we later named “Transcript Scanning” to discover efficiently new alternatively spliced sites in the human CA3N1A transcript. Initially, I wanted to characterize the newly identified CaV2.1 splice variants in Bill’s laboratory but his group at that time no longer conducted experiments in patch-clamp electrophysiology. Even though I was co-author in one of David Yue’s publications, I have not met him before. Nonetheless, I decided to contact him to discuss about possible collaboration on my proposed work. Dave was very generous and immediately agreed to train me in patch-clamp electrophysiology in order for me to characterize the variants I have constructed. As the exon pair 43/44 was found close to the sites for Ca2+-dependent modulation, I decided to investigate the effects all 4 possible combinations of the presence and/or absence of the exons 43 and 44 might have on Ca2+-dependent inactivation and facilitation.

What I discovered was that alternative splicing at this site has profound influence on current density and as a result produced pronounced Ca2+-dependent inactivation (CDI), especially by the A43Δ44 splice variant of the CaV2.1 calcium channel. This observation was contrary to what the field thought which was that CaV2.1 channels did not undergo CDI. For the evaluation of CDI of the paralogous CaV1.2 channels, a 300 msec test pulse was used and under this protocol the reference CaV2.1 channels did not show appreciable or obvious CDI. However, the CaV2.1Δ43Δ44 splice variant consistently demonstrated robust CDI, especially when the current density was large. This observation prompted David, Carla DeMaria and I to look closely at Ca2+-dependent regulation of the P/Q-type CaV2.1 channels using a one second test pulse protocol used by Amy Lee in Bill Catterall’s laboratory. The search for the mechanism confirmed earlier discovery concerning CDI of CaV1.2 channels that calmodulin acts as the Ca2+-sensor. The findings of Carla however clearly showed that the 2 lobes of calmodulin play specific roles: the N-lobe is important for Ca2+-dependent inactivation, while the C-lobe for Ca2+-dependent facilitation (CDF). The demonstration of the functional bipartition of the calmodulin molecule indeed adds to the rationale for the bi-lobe design of calmodulin. What is little known is that Carla and I have done numerous pharmacological and genetic experiments to exclude the role of kinases or phosphatases in determining CDI or CDF. Each time we produced data for an experiment we will print them and then paste the sheets of paper on the wall for the 3 of us to gaze, to analyze and to discuss the next experiments that we would do. I can still image the wall covered with many printed pages of results and the intense anticipation and hope about what the results of each experiment would yield in contributing to the better understanding of Ca2+ signaling by these channels.

Upon returning to Singapore in 2000, aided by a 3-year travel award from JHU-Singapore, I continued to collaborate with David returning to the John Hopkins for 2 weeks each year over the next 3 years to complete some work that we have started in 2000. My fond memories of that collaboration was that Dave’s group would prepare the HEK 293 cells transfected with the required cDNAs for me such that when I set foot into the laboratory on Monday after I have arrived in Baltimore over the weekend, I would be able to do my experiments immediately and there was no time to adjust to jet-lag. The sense of urgency and excellent collaborative and camaraderie spirit characterized the work ethos of Dave and his group. I always enjoyed the intensity of the research atmosphere, and the time spent battling over ideas, generating and refining hypotheses.

Throughout the last 14 years, we have continued to collaborate though I did not visit Dave’s laboratory at the Johns Hopkins anymore after 2004. Through our collaborations, we discovered a molecular switch in the mutually exclusive exons 37a/b that code for part of the EF-hand like domains that were shown to determine whether the CaV2.1 channels exhibit Ca2+-dependent facilitation or not. The greater complexity of Ca2+ regulation by the Cav2.1 channels was unravelled when the alternatively spliced exons were functionally characterized in combination, both in HEK 293 cells and also in neurons. Dave’s group showed that Cav2.1 channels containing the non-facilitating 37b exon can display CDF when it experienced a global rise in cytoplasmic Ca2+. The many levels of Ca2+ homeostasis may have great impact on short term plasticity as well as in disease. To relate to possible physiological or pathological role of alternatively spliced Ca2+ channel exons, we have assessed the relative distribution of exons 37a and 37b in human fetal and adult brains and in different regions of the human adult brain. The results suggested a developmental switch in the usage of exon 37b to 37a and it was more pronounced in the thalamus and substantia nigra; but with little change in the amygdala in which the expression reflected more the fetal expression. Using human cerebellar tissues from people of age ranging from 16–93, we were able to demonstrate that in males, over age, there was a reversal in the switching to a more predominant expression of exon 37b at between ages 30 to 40 years. In the female, the switch occurred at an earlier undetermined age. What was interesting was that in the cortex, the opposite expression was detected. Such developmental change in expression of exon 37a/b was not seen in aging rats or mice. The physiological importance of CaV2.1 CDF has been described, but it will still be interesting to be able to assess behavioral changes in mice devoid of exon 37a or 37b.

Alternative splicing of L-type CaV 1.2 Ca2+ channels in the cardiovascular system

Since 2002, our group has embarked on the search for the diversification of L-type CaV1.2 channel function through alternative splicing. Employing the transcript-scanning technique, we were surprised to identify 19 sites in the CaV1.2 that were subject to alternative splicing. This potentially could generate 2^{19} possible combinations of CaV1.2 splice variants to allow an enormous flexibility for fine-tuning of channel function to cater to physiology and disease conditions. Recently, additional alternatively spliced sites have been reported. The search for tissue-selective splice variant revealed alternatively spliced exon 9* that is expressed selectively in arterial smooth muscle but not in cardiac muscle or in neuron. Alterations in levels of expression of splice variant were observed in pathological conditions of the
of cardiovascular system. It was reported that the expression of exon 9* was increased, while exon 33 was decreased in the surviving cardiac myocytes in rat myocardial infarction, and altered channel property was suggested to be an adaptation to hypoxic conditions for the survival of the cardiomyocytes.

Through profiling of cloned full-length CaV1.2 cDNAs, the analysis of the combinatorial assortment of the alternatively spliced exons yielded 2 predominant cardiac-specific and 4 smooth muscle-specific signature combinatorial profiles (Tang et al, JBC, 2004). Of the 4 smooth muscle splice isoforms, we demonstrated by patch-clamp electrophysiology that a hyperpolarized shift in steady-state inactivation was correlated with increased sensitivity to DHP blockade. This represents potentially a third mechanism to explain vascular selectivity for DHP antagonistic actions against CaV1.2 channels. The other 2 mechanisms include different protein structures due to the inclusion or exclusion of mutually exclusive exon 8 or exon 8a, and that the resting potential of native smooth muscles is more depolarized than cardiac muscles.

Besides alternative splicing, binding of cytoplasmic proteins could modulate CaV1.2 channel properties in a splice variant selective manner. In this regards, Galectin-1 which is a lectin, modulates CaV1.2 channels by binding to the ER export signal found in the cytoplasmic I-II loop. More interestingly, it will interact only in the absence of the alternative exon 9*, and galectin-1 is expressed in smooth but not in cardiac muscle. It is therefore of note that exon 9* not only alters the electrophysiological and pharmacological properties of CaV1.2 channels but it also modulates the interaction between the channel and an interacting cytoplasmic protein such as galectin-1. Given the vascular selective expression of galectin-1, there may be a possible role for galectin-1 to play in hypertension.

One of my concerns when I was establishing the patch-clamp method in my laboratory was whether we have set up the equipment correctly. This problem was more acutely felt as there was no researcher who used the patch-clamp method for ion channel research in Singapore. In the initial years, I would get my postdoctoral fellow to do the first set of electrophysiological recordings of the CaV1.2 channel splice variants using our set-up in Singapore. I will then travel to Baltimore to use the same clonal to conduct the same experiments using David’s rig in what could be considered single blind experiments. I would then compare the results to provide confidence to our group that we have correctly set-up the patch-clamp rig and was able to reproduce our data by 2 experimenters using 2 different electrophysiological set-ups in 2 countries. Before I left Baltimore in 2000, Dave actually sketched the layout of the patch-clamp rig for me to highlight the important electrical connections and to show how best to ground the equipment placed within the patch-clamp rig (Fig. 1). I still kept the copy of the sketch as it meant a lot to me as it reminds me that Dave cared that my electrophysiological work should be up and running as soon as my laboratory was organized to conduct our first experiments in electrophysiology.

**Alternative splicing and RNA editing of L-type CaV 1.3 Ca\(^{2+}\) channels**

Several groups including ours have been interested to determine the generality of alternative splicing as a vehicle to define and optimize Cav channel function in the nervous and cardiovascular systems. One project we collaborated with Dave’s group is to examine the expression of Cav1.3 channels in the auditory hair cells in chick and rat. The rationale for this choice lies with the knowledge that more than 90% of ICa is contributed by the Cav1.3 channels and it was shown that mice deleted of the Cav1.3 gene were deaf and displayed cardiac arrhythmia. Besides the critical role Cav1.3 channels play in hearing, an intriguing observation was the lack of CDI of Ca\(^{2+}\) currents flowing through native cochlear Cav1.3 channels while there was marked inactivation of Ca\(^{2+}\) currents through heterologously expressed Cav1.3 channels. Our hypothesis was that alternative splicing at the C-terminus may produce a splice variant that lacked CDI and the focus was on the exon 41 that codes partially the IQ-domain and its flanking exons. The basis was that the IQ-domain at the C-terminus of the Cav1.2 channels has been shown to be important for Ca\(^{2+}\)-calmodulin binding to mediate CDI. Indeed the alternate use of exon 41 acceptor sites generated a splice variant that because of the use of an alternate splice acceptor site removed the N-terminal portion of exon 41 and because of frameshift also deleted the c-terminal portion of exon 41. The splice variant coded for a Cav1.3 channel that was truncated at the C-terminus and that lacked the IQ-domain which we appropriately named as CaV1.3IQa. These Cav1.3IQa channels were shown to exhibit no CDI and this process was independent of the B-subunit used. The Cav1.3IQa channels were determined by immuno-labeling to be preferentially localized on the outer hair cells (OHCs). The lack of focal expression of the Cav1.3IQa channels suggests the potential contribution of these channels to both neurotransmitter release and motility of the OHCs. This work was done in collaboration with Paul Fuchs in which Dave and I were privileged to be co-Investigator on Paul’s NIH grant.

The laboratories of David Yue and Amy Lee also demonstrated that buffering by calcium binding proteins (CaBPs) could also play a significant role to slow CaV1.3 CI in hair cells. 

We have also reported on our work to “transcript-scan” across the CaV1.3 open reading frame to discover more splice variants and indeed they are more such variations at the C-terminus. Alternative splicing at the C-terminus regulate CDI through elimination of the Distal C-terminal Regulatory Domain (DCRD), reducing the distance between the DCRD and the Proximal C-terminal Regulatory Domain (PCRD) or completely removing exon 41 that codes for the IQ-domain. Xiaodong Liu from Dave’s laboratory has shown that the DCRD interacted with the PCRD to compete out CaM binding to the IQ-domain in order to abrogate CDI. 

Interesting this competition is detected in the human CaV1.3 channel and not rodent channels due to a single valine to alanine switch within position 47 of the CDI-inhibiting module (ICDI). The Strissnig’s group has observed such a phenomenon in their human clone but for those who conducted experiments with the Lipscombe’s rat clone, the rat long-form CaV1.3\(_{42}\) clone did produce robust CDI.
Besides alternative splicing altering CaV1.3 channel electrophysiological properties, we have shown recently the mechanism for influencing DHP-sensitivity in CaV1.3 is completely different from that exhibited by the CaV1.2 channels. We discovered that the mutually exclusive exons 8/8a have no effect on CaV1.3 DHP-sensitivity while instead the binding of calmodulin (CaM) influenced DHP sensitivity. We also showed recently that a compound, 1-(3-chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-(1H,3H,5H)-trione, also known as compound 8 displayed isoform and splice variant selectivity in inhibition. The possible efficacies of Cpd8 and related compounds to selectivity inhibit CaV1.3 over CaV1.2 are still controversial and require clarifications.

Eight years ago, we made a discovery that the IQ-domain of the L-type CaV1.3 channels could be RNA edited at 3 sites. We were extremely excited about it as it was the first report of functional RNA editing of a mammalian voltage-gated calcium channel. Secondly, we also showed that RNA editing was CNS-specific as CaV1.3 channels expressed in peripheral tissues were unedited. Thirdly, edited CaV1.3 channels demonstrated slowing of Ca\textsuperscript{2+}-dependent inactivation (CDI) as shown by whole-cell electrophysiological recordings of edited and unedited CaV1.3 clones expressed heterologously in HEK 293 cells. To interrogate physiological significance, brain slice recordings of the neurons from suprachiasmatic nucleus (SCN) showed that lack of editing decreased the frequency of firing of both the Na\textsuperscript{+} and Ca\textsuperscript{2+} spikes. Fourthly, editing did not influence the subcellular localization of the CaV1.3 channels and edited CaV1.3 channel proteins could be identified by mass spectroscopy to contain the IQ-domain edited amino acid residues. In this work, Dave’s help was sought to use computational method to predict the firing frequency in the SCN with the presence or absence of edited and unedited CaV1.3 channels. He also wrote a script on Matlab to help us analyze the spike frequencies from slice recordings of the SCN done in my laboratory.

David Yue and I collaborated very closely on our projects with regards to the L-type Ca\textsuperscript{2+} channels. Dave was the consummate biophysicist and he was very interested in delineating the molecular mechanisms and underpinnings of CDI of not only the L-type but also the CaV2 family of channels and to uncover any unifying
principle that applies across the various Ca\(^{2+}\) channels that can display CDI.\(^3\) For our group we have interest to determine the physiological importance for altering an ion channel function via alternative splicing or RNA editing and as such we progressed to interrogate such relevance using knock-out mouse technology or relating to human diseases or disorders. This complementarity of our 2 groups’ approaches aided our collaboration and Dave’s deep mechanistic understanding helped us in our interpretation of physiological results. One example is the question of how editing of the Cav1.3 channels slow CDI. Intuitively we investigated the binding affinity of Ca\(^{2+}\)-CaM to the unedited and edited IQ-domain but the results did not pan out. Instead, it was the reduction in binding affinity of ApoCaM that was mediated by editing that slowed CDI.\(^3\)

**Reflections by Masayuki X. Mori, Japan**

In 1999, the Calcium Signals Lab (CSL) in Johns Hopkins University reported the interesting experiment on how over-expressing mutant calmodulin (CaM\(_{1234}\)) eliminated calcium-dependent inactivation in the voltage-gated calcium (Ca\(_V\)) channels.\(^2\) Several other laboratories also reported on the critical role of CaM molecule in regulating Ca\(_V\) channels.\(^4\) At that time, I was a Ph.D. student in the National Institute of Physiological Science in Okazaki Japan and I was very fascinated by these studies, because I was struggling to understand CaM regulation of the neuronal sodium (Na\(_V\)) channels. After finishing my PhD study, I joined the CSL in 2001 as a post-doctoral fellow and I stayed in Dave’s laboratory for 6 years. The six years as a post-doctoral fellow may seem like a long period of time to spend in one laboratory, however, it was quite a meaningful time for me as I contributed to several projects including the structure-function of Ca\(^{2+}\)-dependent facilitation of P/Q-type Ca channels.\(^5\) However, my first project concerned a simple question I asked on how many CaM molecules it might take to turn off a Ca\(_V\) channel and how many CaM molecules were localized around the channels? These “counting questions” seemed very simple but it was not trivial in practice to prove. Amazingly, we could answer these questions using a simple approach in which Ca\(_V\) channels were fused to CaM through a linker. We engineered Ca\(_V\).2-CaM fused channel using several lengths of glycine linkers (Gly2 to Gly12). CaM fused protein naturally exists as a kinase in plant\(^3\) and was successfully used for structural study by generating a CaM-MLCK derived peptide (M13) hybrid molecule, as reported by the Ikura’s group.\(^6\) Such hybrid proteins have a stoichiometry of 1:1 for CaM fused to the kinase or for CaM and target molecule such as MLCK. The original reason for the construction of the fusion channels was that they could be useful proteins for biochemical or structural studies such as to be used for protein crystallization. If this fusion protein were to demonstrate similar functionality as the separate molecules, we would then not need to purify the 2 proteins separately (for example, CaM and IQ-domain of Cav channels). As such, only one CaM fused protein might be good enough for protein crystallization. It cannot be stressed enough that counting CaMs did not feature in our initial planning. Nevertheless, our first result demonstrated that CaM\(_{1234}\) fused channels eliminated Ca\(^{2+}\)-dependent inactivation in Ca\(^{2+}\) channels. Following, we quickly decided to change the direction of our project to the counting of CaMs. After several series of experiments, we have concluded that only one CaM was necessary and sufficient to produce normal CDI.\(^3\) It was the words of David Yue: ‘one bad apple spoils the pie’. Thereafter, crystallographic and biochemical studies revealed that CaM could at least bind at 2 other sites including the C-terminal domain of Cav1.2 channels\(^3\) and the N-terminal region called NSCaTE.\(^4\) Thus, the gap of knowledge between functional counting of CaM and these latter results is still unresolved.

For the second question which is the counting of CaMs at the nano-domain of Ca channels, we were inspired by the tethered blockers of potassium channels reported by Blaustein and Miller’s group.\(^4\) They synthesized a series of quaternary ammonium (QA) pore blockers of varying length that function as molecular tethered blockers of potassium channels. Using Flory’s polymer chain statistics theory,\(^4\) they measured the distance between the tethered point to the pore blocking domain within an angstrom resolution.\(^4\) In our case, we adopted this theory to simulate the concentration of fused mutant CaM with the extended glycine linker (Gly24 to Gly72). The calculation revealed a squeezed endogenous CaM into the CaM\(_{1234}\) fused Ca channels for the re-emergence of CDI, which is 2.4 mM.\(^3\)

Why is it that we could alter the project direction for a new target that we did not imagine? One of the reasons was that David Yue really enjoyed extending the research of Cav channels by learning from different fields and disciplines of science. He was an elegant biophysicist, but equally he had a deep background in physiology, electrical engineering, computation, and he was also equally good in molecular biology, biochemistry, literary writings, music and so on. I think that he was a very rare researcher who could navigate into many fields of science at very high levels, and coupled with creativity, David was able to uncover very interesting mechanisms on how Cav channels work.

David was also very hard working and he never stopped putting immense efforts to ensure the data his group obtained was robust. Hereafter, there will be many more researchers who will embark on research in ion channels, but David will remain a rare talent and uniquely so. Working in CSL was extraordinarily valuable for me, and for all who have worked closely with David, we will continue to work on the science bearing in mind all lessons we have learnt from him. Thank you David!

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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