Journal of Alzheimer's Disease 16 (2009) 909–917
DOI 10.3233/JAD-2009-1026
IOS Press

Discussion

Alzheimer Research Forum Live Discussion: Calcium in AD Pathogenesis

http://www.alzforum.org/res/for/journal/detail.asp?liveID=171

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Note: Transcript has been edited for clarity and accuracy.

Kevin Foskett: Kishore, why do you think there is no neurodegeneration in the Alzheimer's mouse brain despite chronic high Ca^{2+} varicosities and blebs?

Brian Bacskai and Kishore Kuchibhotla: Kevin, we have found that the moderate calcium overload can persist for quite some time, suggesting that even though the dendrite might not be functioning properly, it has not completely degenerated. In addition, we have no evidence that this leads to cell body loss in the neurons– a sort of dendritic pruning.

Tom Fagan: We had one question submitted by e-mail: "Does Ca^{2+} not compete with heavy metals such as cadmium or lead, and if so, what would the consequence be in AD pathogenesis?" Anyone want to field that one?

Ilya Bezprozvanny: Cadmium and lead have much higher affinity for calcium channels than calcium (1,000-fold), so Ca^{2+} is not likely to compete them out.

James Moyer: Kishore, are you able to visualize dendritic degeneration (e.g., in response to challenge) using your *in vivo* approach? And does this susceptibility differ in transgenics?

Brian Bacskai and Kishore Kuchibhotla: James, are you referring to dendritic degeneration in a non-transgenic mouse or in response to an acute lesion?

James Moyer: Kishore, in response to glutamate challenge or some other acute insult.

Brian Bacskai and Kishore Kuchibhotla: James, we can see dendrites respond rapidly to high glutamate concentrations but have not compared that carefully to transgenic mice.

Beth Stutzmann: Kishore, I think you partially addressed my follow-up question to Kevin's, but have you looked further down into the cell body regions to determine extent of calcium overload?

Brian Bacskai and Kishore Kuchibhotla: Beth, we have not fully characterized the cell bodies in layer 5 just yet.

ISSN 1387-2877/09/$17.00

Tom Fagan: Everyone, a general question that was raised by Kim, I think: what is going on upstream? How do presenilins (PS) affect so many different calcium channels?

Beth Stutzmann: Tom, I do not think PS affects all calcium channels equally; for example, I see no effects whatsoever on spike-evoked calcium entry, suggesting that L-type channels are not affected. There is more evidence, although scattered, that amyloid-β (Aβ) affects calcium channels (e.g., downregulates voltage-gated P/Q calcium channel).

Kim Green: Beth, absolutely, which is why I think that presenilins are mediating their effects at the endoplasmic reticulum (ER) level.

Kevin Foskett: Tom, I think that what will be critical to distinguish is between primary and secondary mechanisms. A primary defect in one pathway [e.g., hyperactivation of the inositol 1,4,5-triphosphate (IP_3) receptor by mutant presenilins] may affect transcriptional events and cell bioenergetics that could lead to secondary aspects of calcium signaling dysregulation. I personally do not think that the multiple mechanisms described will (at the end of the day) be determined to be primary mechanisms.

Tom Fagan: Kevin, do you think PSs are interacting directly with the receptor, then?

Kevin Foskett: Tom, co-immunoprecipitation experiments are consistent with an interaction of both wild-type (WT) and mutant presenilins (mPS) with the IP_3 receptor. The electrophysiological results are most consistent with a direct interaction, but we have not demonstrated that using biochemical approaches, and the molecular basis of the co-immunoprecipitation is still unclear.

Ilya Bezprozvanny: Kevin, I am surprised you see effects of IP_3 R-mediated ER leak in permeabilized DT40 cell preparation. Where does IP_3 come from in these experiments?

Kevin Foskett: Ilya, we believe that IP_3 is either retained inside the permeabilized cells or is produced locally. Indeed, unpublished data from our laboratory suggests that IP_3 can be generated in the nuclear envelope. Therefore, in the endoplasmic reticulum, more generally.

James Moyer: Beth, in your triple transgenics, what happens to induction of long-term depression (LTD) in the presence of dantrolene? Is it exaggerated or affected in any way?

Beth Stutzmann: James, we are running those experiments right now, as a matter of fact. Ask me again in a week or so.... We are expecting to see differences between non-transgenic and 3xTg, particularly since LTD requires the recruitment of ER calcium stores.

Tom Fagan: Carlos Villalobos also had some interesting questions. Carlos, do you want to address those?

Carlos Villalobos: Thank you, Tom. I will try. A little bit of Aβ now. I would like to know, according to the opinion of the panel, what is the main suspect pathway for Aβ-induced entry of calcium into neurons?

Brian Bacskai and Kishore Kuchibhotla: Carlos, there are many possible entry points, including 1) Aβ as a calcium channel, 2) via NMDA channels, and 3) via voltage-gated calcium channels. It will be possible to differentially test these possibilities using pharmacology and imaging approaches.

Carlos Villalobos: Brian and everyone in the audience, Nelson Arispe came up more than a decade ago with the amyloid channel, but still there is no consensus on that. What do you think?

Brian Bacskai and Kishore Kuchibhotla: Carlos, the amyloid channel is an intriguing hypothesis, and we think the preparations we have is well suited to test it. A key issue is whether we can reliably and selectively block an Aβ channel *in vivo*.

Ilya Bezprozvanny: Carlos, I think Aβ channels described by Arispe are critical for Aβ-induced Ca elevation. It is also important that these channels form more easily if cells express phosphatidylserine on the surface; please see detailed discussion in TINS review of this idea [1].

Brian Bacskai and Kishore Kuchibhotla: Carlos and Ilya, one critical point about Aβ and calcium overload is that not every dendrite responds to Aβ in the same way. What makes a given dendrite susceptible to the Aβ-induced overload compared to another dendritic process?

Ilya Bezprozvanny: Brian and Kishore, if I have to speculate I will argue that dendrites that have more phosphatidylserine on the surface are more susceptible for "amyloid attack" [2].

James Moyer: Ilya, is there any evidence that when Aβ forms a calcium pore in the membrane, it is preferentially linked to ER sources of calcium, or other cytoskeletal proteins or kinases, etc.?

Kevin Foskett: Ilya, ER leak function might predict that downstream events from normal ER Ca^{2+} leak signals would be inhibited, whereas hyperactivity of the IP_3r or ryanodine receptor (RyR) might be expected to enhance those pathways. These ideas can be tested in *in vitro* and *in vivo* models.

Kinga Michno: I am interested in knowing what the take is on data generated by Cristina Fasolato's group [3] demonstrating that the ER calcium (and Golgi) calcium levels are decreased when familial Alzheimer's disease (FAD) and in fact WT PS are expressed. It seems to me that a more logical consequence to increased calcium channel activity would be decrements in internal stores. I would appreciate hearing the panel's thoughts on this.

Kevin Foskett: Kinga, our results also show a diminished ER Ca^{2+} store in agreement with Fasolato. We suggest it is due to hyperactivity of the IP_3 receptor.

Ilya Bezprozvanny: Kinga, most data point to elevated Ca^{2+} content in ER stores in FAD cells. From our experience murine embryonic fibroblast (MEF) cells tend to "drift" a lot, so the ER Ca^{2+} content changes a lot with passage number. We now confirmed elevated ER Ca^{2+} level in primary hippocampal neuronal cultures from PS-FAD mutant mice.

Kinga Michno: Ilya, I am scrambling to find the reference (unsuccessfully at the moment but I will keep looking), but perhaps you will be familiar with much earlier work in PS1/PS2 double knockout cells which demonstrated that these cells actually exhibit decrements in internal stores content (using thapsigargin, I believe). Any comments?

Ilya Bezprozvanny: Kinga, yes, Cell Calcium paper from De Smedt's group [4]. Bottom line is that MEF cells "drift" and different laboratories use different passage number. Also, thapsigargin is not a good way to determine store content, as it depends on the leak.

Kinga Michno: Ilya, one last question for you. In your experimental approach describing PS's passive calcium channel activity, can you be certain that in your method of isolating PS protein for your experiments you did not also pull down a calcium channel (since at least a couple have been shown to bind to PS).

Ilya Bezprozvanny: Kinga, we affinity purified His-PS1 on Ni column and reconstituted in liposomes and planar lipid bilayers to rule this out [5].

Ivan Goussakov: Brian and Kishore, how do you really separate cell compartment soma, dendrites, and spines while using recently available time resolution?

Brian Bacskai and Kishore Kuchibhotla: Ivan, you can use the structure of the neuron to differentiate the soma, dendrite, and dendritic spine. What you see is what you can resolve.

Craig Atwood: An open question. I do not think anyone would disagree that Ca^{2+} plays a role in Alzheimer's and other neurodegenerative diseases. Is Ca^{2+} a downstream mechanism, induced by changes in AβPP/presenilin metabolism? And if so, then whatever regulates AβPP and presenilin metabolism would ultimately be regulating AD biochemical, pathological, and cognitive changes. In this respect, are drugs that target Ca^{2+} metabolism really only acting as a band-aid for upstream causes of the disease?

Ilya Bezprozvanny: Craig, the question of amyloid and calcium – upstream or downstream – is *the central* question in my opinion. I do not think we know if it is abnormal calcium driving amyloid pathology or amyloid pathology driving calcium. Data support on *both* so far.

Beth Stutzmann: Craig, I think calcium has an equal opportunity role to act as an early initiator of AD pathogenesis (as in FAD cases), and to accelerate existing histopathology and cognitive deficits (as in sporadic) – depending upon where/when in the cycle it enters.

Kevin Foskett: Craig, it is very compelling to me that calcium signaling disruptions are observed in a wide variety of cell types in a wide variety of laboratories upon expression of mutant presenilins. To me, this suggests a fundamental, proximal defect that could be upstream of pathology in AD.

Craig Atwood: Kevin, I absolutely agree that mutant presenilins can affect calcium metabolism and be a downstream mechanism driving FAD. But what is driving the sporadic forms of the disease? What are the age-related changes regulating Ca metabolism (and amyloid metabolism/PS1)?

Brian Bacskai and Kishore Kuchibhotla: Ilya, with changes that you see in Ca^{2+} leak, how do you think the neuron (or neural system) would respond during intact brain development? Is it possible that the increased leak would be masked by some compensatory mechanisms?

Ilya Bezprozvanny: Brian and Kishore, absolutely. There are many compensation mechanisms in case of reduced ER Ca^{2+} leak. I think increased expression of RyR in aging PS1-M146V KI mice as observed by Beth is one of them [6]. Neurons try to reduce their ER Ca^{2+} levels back to normal range, as ER stress response is activated when ER Ca^{2+} is too high.

Craig Atwood: Ilya/Beth, irrespective of whether it is Ca^{2+} or Aβ, would you agree that upstream mechanisms/causes are what regulate amyloid and Ca^{2+} metabolism in the first place? Amyloid, for example, does not just drop out of the sky into the brain! And I suspect the same for Ca^{2+}. So something upstream has to be regulating Ca^{2+} and amyloid metabolism, or both. Does Ca^{2+} metabolism change during cell division? There is a lot of evidence that pyramidal neurons in the AD brain have re-entered the cell division cycle and are attempting to divide.

Beth Stutzmann: Take, for example, the AβPP/tau mice, which do not show early calcium signaling defects but will generate plaques. I think calcium deficits will be downstream of this. Look at the PS mutations, and calcium far precedes AD pathology. Interestingly, I see similar levels of calcium dysregulation in mPS1 mice and 3xTg mice, up until $\sim$12 months when there is significant plaque/tangle pathology. At this late pathology stage, there are even greater calcium responses in the 3xTg.

Kim Green: Craig, the development of sporadic AD is complex, and risk for the disease can be increased by a wide variety of events, but an aged brain is essential. Calcium may not be initiating the disease in all cases, but may play a role in some.

Ilya Bezprozvanny: Craig, there is ample literature on aging-related changes in neuronal calcium signaling. It seems to be generally consistent with enhanced Ca^{2+} signaling as neurons age. We cite some of these in the TINS review. My general feeling is that increased Ca^{2+} signaling is a normal result of aging, but in AD it is accelerated (or more damaging to neurons).

Kevin Foskett: Craig, the FAD mutations provide clues that Ca^{2+} could be involved in sporadic AD. The same molecular mechanisms involving PSs and the IP3 receptor associated with FAD may be different in sporadic AD. For example, different Ca^{2+} regulatory proteins may contribute to Ca^{2+} homeostasis dysfunction in sporadic AD. Indeed, identification of CALHM1 as a risk factor for sporadic AD and its role in plasma membrane Ca^{2+} permeability is consistent with such a notion.

Kim Green: Beth, a transgenic mouse is preprogrammed to develop pathology due to the transgene promoters used. Therefore, they are useful for looking at the downstream effects of pathology, but not so useful for understanding what causes the pathology in the first place. We need models of sporadic AD, in which we can modulate calcium pathways and evaluate the effects on pathology.

Beth Stutzmann: Kim, true, but that was largely my point. I would expect to see downstream calcium effects in the AβPP/tau mice which can be linked to the histopathology (as I do in the aged 3xTg mice), but make no assumptions about how the histopathology got there. Regarding more sporadic models being needed, I could not agree more. There are apolipoprotein E4 (ApoE4) mice, but as far as I know, there are few calcium studies on them. It is known that ApoE4 expression will result in increases in calcium, though.

Craig Atwood: Ilya/Kevin, I agree. But the question still remains as to what age-related changes are regulating Ca^{2+} changes in the brain. Yes, they occur, but what is the mechanism? I know, for example, that reproductive hormones (sex steroids, gonadotropins, GnRH, activins) can regulate the cell cycle and also amyloid production and tau phosphorylation. Do these hormones affect Ca^{2+} metabolism in the brain? Is anything known about how hormones may regulate Ca^{2+} metabolism in the ovary or testis?

Kevin Foskett: Craig, your questions are good ones. However, one could look at it from the opposite point of view, and ask, What is it about aging that may make the

brain more susceptible to damage by perhaps normal calcium signaling mechanisms?

Craig Atwood: Kevin, thanks. Yes, we did look at this angle [7], hence my questions on the cell cycle and how aberrant re-entry into the cell cycle may affect how a cell responds to Ca^{2+}.

James Moyer: Craig, we have seen similar increases in afterhyperpolarization (AHP) and enhanced frequency accommodation in aged rabbit CA1 neurons; these effects were differentially reduced by nimodipine in aged neurons.

Beth Stutzmann: Craig, I would refer you to Phil Landfield's and Oliver Thibault's studies on changes in calcium with aging: basically, what they find is an upregulation in L-type calcium channels which are linked to increased IcmF associated homologous proteins (IAHP) currents, which serve to reduce membrane excitability [8].

Volodymyr Rybalchenko: Oxidative stress is generally increased in cells of aging organisms.

Carlos Villalobos: Kishore, how can calcineurin promote further calcium increases?

Brian Bacskai and Kishore Kuchibhotla: Carlos, great question. This result surprised us, but there is at least a bit of literature supporting this in a reduced preparation (but still no mechanistic insight).

Tom Fagan: Everyone, do we have any consensus as to which Ca^{2+} ER channel might be most important from a quantitative viewpoint? I imagine it might be difficult to compare across methodologies, but is there any indication as to which channel response is most robust?

Beth Stutzmann: Tom, the data are still quite preliminary on my end, but I am finding that when I evoke an IP_3 response (with caged IP_3) or a RyR response (with caffeine), I am not really getting a "pure" channel response. When attempting to isolate either the RyR or the IP_3R, I have found that I get a much larger mixed calcium response in the mPS neurons compared to non-transgenics, so that CICR seems upregulated/sensitized. But, this effect is much larger and extends throughout all neuronal compartments for the RyR activation.

Brian Bacskai and Kishore Kuchibhotla: Tom, I think your question is an important one. What is the magnitude of the calcium alterations based on ER stores refilling or leaking? How does this affect function? The difference between resting and dynamic changes in calcium may start to address this.

Kevin Foskett: Tom, there will not be a consensus regarding the importance of a particular ER Ca^{2+} permeability until the published studies are reproduced in other laboratories, and until the very different hypotheses that the various mechanisms predict are tested.

Carlos Villalobos: Brian/Kishore, that is difficult to say. Among multiple things, a chance is that a cytosolic calcium overload depends not only on local calcium entry but also on local calcium uptake by, for instance, surrounding mitochondria.

Craig Atwood: Beth, how does ApoE4 affect calcium? I know ApoE4 carries cholesterol into cells for use in membranes and conversion to sex steroids. Do sex steroids alter Ca^{2+} channels?

Beth Stutzmann: Craig, I am going to cut and paste from my 2007 review to save time: The mechanism by which ApoE4 alters intracellular calcium is thought to involve a cell-surface LDL receptor mediated process. The increased calcium influx from extracellular sources most likely reflects activity-dependent activation, and not a steady-state upregulation, which would too quickly become neurotoxic. I believe the mechanism is still unclear, but can again refer you to the primary literature [9–13].

Shreaya Chakroborty: Volodymyr, your studies have shown that wild-type PS increases the open probability of RyR channels. Do you know if mutant PS keeps the RyR locked in an open configuration similar to the effect it has on the IP_3R?

Volodymyr Rybalchenko: Shreaya, we did not study the effects of mutant PS. Also, we tested the effects of the soluble cytoplasmic terminus of PS. There are almost no mutations found in cytoplasmic terminus related to AD. Our mechanism could probably play a role in the occurrence of sporadic AD.

Tom Fagan: I would like to throw the perpetual "what is next?" question. What are the crucial experiments that need to be done? Are there technical chal-

lenges? Funding challenges? In an "anything is possible world," what would you look at next?

Brian Bacskai and Kishore Kuchibhotla: Tom, one "what next" question is, What seeds an individual plaque to deposit where it does? Is calcium related to that pathological event or is it completely downstream?

Craig Atwood: Brian and Kishore, there is a whole literature on this that includes metal ions (Cu, Zn) and pH changes as the nucleating factors [14,15].

Tom Fagan: Brian/Kishore, so how would you get a plaque to form in culture? Has anyone considered that question?

Brian Bacskai and Kishore Kuchibhotla: Tom, why do we need to go to culture? We can watch *in vivo*.

Tom Fagan: Brian/Kishore, true, but I thought culture would give you more controls over manipulating the system.

Brian Bacskai and Kishore Kuchibhotla: Tom, you cannot make a plaque in culture. Yet.

Kevin Foskett: Tom, the approaches taken by Stutzmann, and especially Brian and Kishore, to appropriate animal models will be the way to get at the pathophysiological relevance of the Ca^{2+} hypothesis generally and any of the proposed mechanisms specifically. This will be expensive because it involves high technology and animals, so the challenge will be for the Alzheimer's Association to raise enough money to fund us well.

Ilya Bezprozvanny: Tom, I think the most important direction is to sort out upstream/downstream issues for Ca^{2+}/amyloid. This is a "chicken/egg" problem that needs to be sorted out, which is not easy due to limitations of available AD mouse models that do not have degeneration, only plaques.

Beth Stutzmann: Tom, I think that approaching this calcium "chicken and egg" problem can also be addressed from the opposite side of the fence. If we can normalize the calcium, will that reduce/prevent/have no effect on AD pathology? We can acutely normalize the exaggerated calcium with dantrolene, but what are the chronic effects? Sounds like a trivial experiment, but it is not.

Ilya Bezprozvanny: Beth, the problem is readout. When you say "AD pathology," what do you plan to look at? Plaques?

Beth Stutzmann: Ilya, yes, very good point. Ultimately, I think people with AD want their memory and minds back, and are not too interested if plaques are there. That would be the ultimate readout. There is still little consensus if Aβ has anything to do with cognitive impairments clinically. Tau is looking like a better histopathological correlate, as is synapse loss. Mice are already pretty cognitively impaired to start with, so using a well-correlated marker might be a better tool.

Craig Atwood: Brian and Kishore, I believe that someone did make plaques in cell culture a few years back, but the name escapes me.

Tom Fagan: Craig, really? Plaques in culture could be very useful, I would have thought. Maybe the process is too hit-and-miss.

Brian Bacskai and Kishore Kuchibhotla: Craig, if we could make *bona fide* plaques in culture, we and almost everyone else would be using them every day.

Craig Atwood: Brian, look up work by Janusz Frackowiak [16].

Tom Fagan: Craig, muscle cells. Wonder if this has been repeated.

Craig Atwood: He has a few papers on this general area, all of which I cannot remember, but this will get Brian headed in the right direction.

Carlos Villalobos: Audience, does anyone know the mechanism of neurotoxicity by Aβ fibrils?

Craig Atwood: Carlos, according to Mark Mattson, by upregulating Ca^{2+} entry into neurons and leading to apoptosis.

Carlos Villalobos: Craig, do you know if additional mechanisms have been claimed for Aβ fibrils?

Craig Atwood: Carlos, yes, look up Liu et al. [17] introduction and last figure for an overview of p25/35 and tau mechanisms. There are oxidative mechanisms, also.

Kim Green: Ilya, while animal models do not have neuronal loss like human AD, they do have significant cognitive decline. This suggests that the pathology influences cognition (probably at the synaptic level), rather than just through neurodegeneration, and gives us a pathway we can explore and then hopefully block.

Tom Fagan: Thanks all for the thoughts on future directions. Beth, is there an alternative to dantrolene?

Beth Stutzmann: Tom, looking into it . . .

Ilya Bezprozvanny: Tom, me, too.

Craig Atwood: Beth, you get at the original question I posited; if you can stabilize Ca^{2+} but still see functional loss, then Ca^{2+} is not the answer. I guess that is the key Ca^{2+} experiment to do.

Beth Stutzmann: Craig, that is a fundamental question (that may force some of us to switch jobs . . .). But yes, it is certainly possible that the calcium dysregulation is an epiphenomenon that has little to do with AD. I do not think this is the case, but perhaps calcium dysregulation is not specific to AD, as similar changes have been reported in Parkinson's disease, Huntington's disease, and other neurodegenerative diseases.

Carlos Villalobos: Beth, it may be that normalizing calcium release/entry is not enough if intracellular calcium buffers are being lost.

Ivan Goussakov: Brian and Kishore, calcium transient in dendrite, which starts from normal glutamatergic synaptic activation, should be dependent on synaptic density. Ca^{2+} diffusion (even diffusion only) cannot be followed up in time with recent Ca^{2+} imaging technology. Is it difficult to differentiate between compartments?

Brian Bacskai and Kishore Kuchibhotla: Ivan, yes, there are limitations on spatial and temporal resolution, but the state of the art is advancing rapidly. We can easily resolve spines from parent dendrites and can scan at kHz rates in subfields if necessary.

Ivan Goussakov: Brian and Kishore, so I will be looking for the future when the scan rates can exceed 1 to 5 KHz per line scan and for fluorophores that will be able to give at least 10 photons per 0.3 ms.

Brian Bacskai and Kishore Kuchibhotla: Ivan, I think I am starting to understand what you are getting at. The limitation for the studies you are proposing is in the dyes themselves. They allow ms time resolution at best. Perhaps voltage sensitive dyes would be more appropriate?

Ivan Goussakov: Brian and Kishore, spines and dendrites need to be separated not only in space (by frame imaging) but in time in order to see the time sequence of Ca^{2+} transients. So can we talk about [Ca^{2+}] difference in compartments while working with averaged images (10–30 ms) of branches covered by spines?

Brian Bacskai and Kishore Kuchibhotla: Ivan, we have not tried yet.

Tom Fagan: Brian and Kishore, Ilya mentioned in his talk about a potential Ca^{2+} influx channel – not sure if that has come up in the discussion here – but do you have thoughts?

Ilya Bezprozvanny: Brian and Kishore, yes, it looks like we all have to start running "memory paradigms" in mice, no way around it.

Brian Bacskai and Kishore Kuchibhotla: Ilya, the behavioral assays can be informative, but have limitations . . .

Ilya Bezprozvanny: Brian and Kishore, what is a good readout, then? Short of a human clinical trial?

Brian Bacskai and Kishore Kuchibhotla: Ilya, all of the approaches in mice or culture (calcium levels, function, structure, and behavior) are helpful . . . but you might be right that the only way to really know if any of these are important in AD is if they actually work in the clinic. We have to remind ourselves that we are working with models.

Ilya Bezprozvanny: Brian and Kishore, that is why I tested Dimebon [18], which was claimed to be very effective in human AD Phase 2 trials [19]. But effects of Dimebon on Ca^{2+} signaling required a 50 μM dose – not likely to be physiological. Instead we found that Dimebon hits a number of receptors – histamine, serotonin, adrenergic. Most likely effects on these receptors are responsible for clinical effects on Dimebon as a "cognitive enhancer" in short-term trials.

Shreaya Chakroborty: Volodymyr, how would you test your hypothesis that your WT PS-RyR mechanism could play a role in sporadic AD?

Volodymyr Rybalchenko: Shreaya, this would be a disclosure of the commercially valuable information.

Shreaya Chakroborty: I understand, but I had to try.

Kevin Foskett: All, a fundamental issue for those of us studying the role of Ca^{2+} signaling in the pathogenesis of AD is to prove to the amyloid field that this is a relevant mechanism(s). A key aspect going forward is to establish the relevance of the Ca^{2+} hypothesis in a convincing manner. Clearly, it seems to me, animal models will be the key. The mouse offers clear benefits, as well as limitations. Is there a prospect for new animal models to complement the mouse?

Kim Green: Kevin, I think that Brian and Kishore's work has shown that calcium is downstream of pathology, and we can work with that on existing models. We will need new models to prove that calcium can influence pathological progression *in vivo*, and I think we are still some time away from that.

Beth Stutzmann: Kevin and all, yes, operative word I think is "mechanism," which is why the single channel studies are so important. Historically, the Aβ group is protein biochemistry based, and has much to learn about channel biophysics and why it is important. I cannot reach that limit of resolution in my studies (nor can Brian and Kishore), but it is critical information that can be integrated with the next level of analysis.

Tom Fagan: All, someone just mentioned the clinic. This might be a very naive question, but are there any known conditions, drugs, etc., that affect calcium homeostasis and could be worthy of study in this context?

Carlos Villalobos: Tom, a few days ago we saw no response (calcium increase) of vascular smooth muscle cells to Aβ oligomers, but they were a cell line, not primary cells.

Tom Fagan: Carlos, "days ago"; that is hot data!

Carlos Villalobos: Is there a better audience to release it to?

Ilya Bezprozvanny: Tom, I mentioned Memantine and Dimebon in my presentation. These are only two clinical drugs tested in clinical AD trials which have a Ca^{2+} connection. Do not know if there are any more that are being tested in clinic.

Carlos Villalobos: Although non-steroidal anti-inflammatory drugs (NSAIDs) are not generally considered as calcium antagonists, in fact there are: NSAIDs that are mitochondrial calcium antagonists and protect both *in vitro* and (likely) *in vivo*.

Tom Fagan: Thanks, all. We look forward to more exciting data soon.

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