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Discussion

Alzheimer Research Forum Live Discussion: Cell Cycle Hypothesis Pedaling into Mainstream Acceptance? Results in Fly, Mouse Models Warrant a Second Look[1]

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Gabrielle Strobel I am Gabrielle Strobel, managing editor of Alzforum, and I will moderate.

Inez Vincent Forgive me for bringing up the issue of whether the neuronal Cdk5 kinase should be considered a "cell cycle kinase" or not.

Karl Herrup I think Cdk5 is very much a cell cycle kinase (I am prejudiced, of course), but it functions to arrest the cycle rather than promote it.

Vikram Khurana Regarding Cdk5, at least in the context of the fly, the genetic modification of tau toxicity differs from cell cycle mediators.

Gabrielle Strobel What do you mean, Vikram? Can you explain a bit more?

Vikram Khurana Gabrielle, my preliminary data would suggest that, while Cdk5 is a good enhancer of wild-type tau-induced toxicity in our system, it does not appreciably enhance the toxicity of our pseudophosphorylated (E14) construct. This is very different from cyclins coexpressed with cell cycle-related Cdks, which strongly enhance both tau and E14-induced neurodegeneration. In our system it thus seems Cdk5 may be working via tau phosphorylation in contrast to cell cycle-related kinases which have a downstream role directly upstream of apoptosis.

Inez Vincent Karl, yours is a good point, but I am worried that a cell cycle kinase is often construed as one that promotes or activates cell division.

Karl Herrup Inez, I think that Cdk5 may be only one of many cell cycle proteins that may turn out to have paradoxical functions in neurons.

[1] Note: The transcript has been edited for clarity and accuracy.

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Gabrielle Strobel I would like to start the discussion off by inviting all our excellent investigators to state in a nutshell what, to their minds, is the single biggest advance in the hypothesis of the cell cycle in neurodegenerative pathogenesis.

Mark Smith The Andorfer paper [1] on the relationship between tau and the cell cycle/cell death.

Inez Vincent I think the best thing that has happened in this field is that we have taken our original speculations to a different level by systematically demonstrating that the cell cycle plays a role in a wide range of *in vivo* conditions.

Xiongwei Zhu In my mind, the recent realization of the relationship between tau and cell cycle disturbance is a major advance.

Vikram Khurana Gabrielle, I am a little biased regarding important advances. But I would like to draw attention to the work of the Bonni and Greene labs in delineating molecular pathways (Cdc2, E2f/chromatin remodeling) that underlie the interaction between cell cycle and apoptotic machineries [2–4].

Gabrielle Strobel I agree, Vikram.

Donna McPhie I think Herrup's laboratory demonstration of tetraploid nuclei in AD brain was a major advance [5].

Mark Smith Inez, I agree, and I think the Andorfer and Khurana [6] papers were key in this respect. The Zhu "two-hit" paper [7] was also an important conceptual advance (though obviously I am not without bias).

Karl Herrup Let us move to Vik's paper. I would like his views on a couple of things. The first is paradoxical. With all the genetics you were able to bring to the project, we still do not know that DNA was actually replicated in the cells. Could you (did you) investigate this?

Vikram Khurana Karl, that is a great question. We did try BrdU feedings on our flies (this has not been done in the adult fly brain before), but without success. Our feeling is that the limitations here were probably technical. Since we considered the major unanswered question in the field was of causality, we focused on this question in our work.

Patricia Estani I would like to ask the author, Vikram Khurana, what is the causality relationship which he refers to?

Vikram Khurana Patricia, I refer to the causal connection between cell cycle activation and neurodegeneration in the context of an animal model of human disease.

Karl Herrup And I do not want to take away from what you did. I thought it was a fabulous paper. You are to be congratulated.

Xiongwei Zhu In relation to Karl's question, since the fly is quite a neat model, is it possible to differentiate what phase these neurons are at before they die?

Cathy Andorfer Vik, I want to congratulate you as well – great study. The rapamycin study is particularly exciting because it has been shown to arrest cells at the G1/S boundary – the point that most of the misexpressed cell cycle factors relate to. I have been planning to do similar studies in transgenic mice; your fly work is an encouraging proof of principle.

Vikram Khurana Cathy, I wanted to ask you what progress has been made on cell cycle inhibition in the tauopathy mouse model?

Cathy Andorfer Vik, on this point I am still working on breaking down the mechanism in mouse models, currently trying to determine if there are differences between mutant versus nonmutant tau in relation to cell cycle abnormalities. Inhibition studies are coming soon.

Karl Herrup But I think the issue of whether DNA replication occurs is an important one. When I talk about the cell cycle in neurons now (especially with our Cdk5 as anti-cyclin work), I feel that I have fallen down a rabbit hole and ended up in Wonderland.

Gabrielle Strobel Karl, what did you see when you landed at the bottom of the rabbit hole?

Karl Herrup Gabrielle, I see cyclin A upregulated like "gangbusters" but localized almost exclusively in cytoplasm. I see E2F1 in dendrites, but moving to the nucleus under stress. I see Cdk5 functioning as a kinase for just about every synaptic vesicle protein I know. And more that I am not prepared to talk about right now.

Inez Vincent Is it possible that cell cycle entry and progression may have an entirely different mechanism in neurons? That is, it may still require some key molecules such as retinoblastoma protein (Rb), Cdc2, Cdk4, but other regulators may be neuronal.

Jin-Jing Pei Vik, do you want to say a bit about mTOR (mammalian target of rapamycin)? We got into mTOR/S6 kinase because of total tau level increase in AD brains [8].

Rachael Neve Yes, I would like to hear more about that pathway and why you focused in on it.

Vikram Khurana Well, the TOR pathway was of interest to us because of all the mitogenic signaling pathways upregulated in AD. It was a relatively downstream and convergent pathway with direct links to the cell cycle machinery. Hariharan's group had also shown strong genetic interactions between the TOR pathway and the cell cycle in *Drosophila* [9], which enabled us to address this question thoroughly. Furthermore, the links of TOR to caloric restriction/aging in a variety of contexts raised intriguing possibilities of connections to neurodegeneration.

Inez Vincent I would like to raise the point about the temporal sequence of events. Karl, Rachael Neve, and others have shown that the cell cycle may be activated by extracellular amyloid, or intrinsic amyloid mutations, and Cathy and now Vikram have shown that changes in tau levels or phosphorylation trigger cell cycle activation. But in AD brain and other conditions, most of us have detected cell cycle changes in neurons known to be vulnerable to the disease, but either without any tau changes, or early changes in tau hyperphosphorylation, which suggested then that cell cycle changes precede tau phosphorylation.

Mark Smith The issue of cell specificity is important to address with respect to chronology; that is, are cell cycle changes phenomena or epiphenomena?

Jin-Jing Pei Inez, I like your original 1997 paper on aberrant neuronal expression of Cdc2/cyclin B1 in AD [10].

Vikram Khurana Inez, it is true. In our model we present multiple lines of evidence indicating cell cycle is downstream of phospho-tau, but there may be additional mechanisms operating in disease and other models.

Inez Vincent I think there is sufficient *in vitro* and *in vivo* data to support the idea that cell cycle changes are essential for neurodegeneration. Whether they are also essential for lesion formation is not as simple to answer at the moment.

Jin-Jing Pei Inez, can you explain more about the neurodegeneration?

Inez Vincent Yes, I meant neuronal death – by any mechanism, whether apoptotic – Vikram, Susan Ackerman [11], David Park [12], Lloyd Greene [13], etc. – or not (Herrup, in the Atm mice [14].

Karl Herrup Another question for Vik. I notice that you looked at the double labeling of phospho-tau and cell cycle. You always express your result as the cell cycle-positive cells were also tau-positive. Was the reverse true? I ask because if you look at our AD study [15], we found that the reverse was much less true.

Vikram Khurana Karl, we found the reverse was not true. In other words, many phospho-tau cells were not cell cycle-positive. We suggest this as supportive immunocytochemical data for our genetic data implicating cell cycle activation downstream of tau phosphorylation.

Karl Herrup Okay, Vik, here is the question. Tau has been proposed as a sink for toxic "stuff" (kinases being only one). You were able to modify tau toxicity with cell cycle proteins, but you could not drive death with cell cycle proteins. To me that suggests that this is not a linear pathway and that we had better be a bit cautious about thinking of the cell cycle as "downstream."

Vikram Khurana Actually, Karl, in Fig. 4 of the paper, we do show that ectopic cell cycle activation does lead to apoptosis in neurons. I am not sure if I misinterpreted your question?

Karl Herrup Vik, you got it right. I missed the point. But there was a lot of negative data as well (cyclin A, if I remember, by itself did nothing, for example).

Daniel Geschwind Vikram, great, impressive paper – huge amount of fly genetics. I was wondering whether there was any evidence that the components of the TOR pathway might contribute to the regional susceptibility in the disease. Karl, I will admit my ignorance as to the

details of your work and also ask the broader question regarding cell cycle and specific neuronal populations that are most vulnerable in AD.

Vikram Khurana Dan, I do not know if TOR in different brain regions is a factor, but it would be very interesting to look at this. Jin-Jing, any thoughts on this?

Jin-Jing Pei Vik, yes. I agree that the later part of the hypothesis involving mTOR is only involving about 30 percent of cell death. Other mechanisms may be involved, also. Do you think S6K represents mTOR activity properly?

Vikram Khurana Jin-Jing, it is certainly a well-established downstream target, but it can be activated in other contexts. Our genetic data, however, using multiple reagents to block the TOR pathway, would implicate this pathway as a whole in tau-induced neurodegeneration.

Daniel Geschwind Vik, here is a specific question about Fig. 5 to make sure I understand the experiments fully. The various TOR antagonists, both pharmaceutical and genetic, give a similar drop in TUNEL staining (25–50 percent), and this is highly significant. However, it is not full block. In your estimation, is this due to methodological issues (i.e., full blockade is asking too much, given the methods) or the activity of other yet unnamed pathways?

Vikram Khurana Dan, while it is always possible that there are multiple parallel pathways working here, our data do not imply that necessarily. In both the TOR inactivation and cell cycle inactivation interventions, we certainly do not block the pathways completely because the fly brains develop normally, so these transgenes only put the brakes on these pathways and do not cause complete inhibition. Therefore, complete rescue would not be expected. I have also expressed tau in the eye in the context of Rheb-/- clones and show almost complete suppression of retinal degeneration. The rescue is not complete, however, implying that other pathways are presumably also activated.

Gabrielle Strobel Karl, I am intrigued by the link to synaptic biology you are now seeing. Do you think the neurons past S phase are surviving for a while, but their synaptic regulation is off, due to rampant Cdk5? Could this accommodate cell cycle activation as an early event, with ensuing synaptic dysfunction, and cell death when the neuron sustains another hit? I would also like to repeat Dan's question about regional subpopulations. Can you remind us if you see cell cycle upregulation and DNA synthesis in specific populations only?

Karl Herrup Dan, Gabrielle, the regional variation is unexplained "for my money". The mouse AD model we are using tracks the human anatomy pretty well. But, as we say, the reactivation of the cell cycle does not lead to death in mice.

Vikram Khurana Karl, we purposely used a modifier (cyclin A) in Fig. 2 that did not cause appreciable apoptosis in the fly brain for the purposes of showing synergy with tau. The differences between the cyclins may be trivial, that is, just related to levels of expression of the particular transgenes.

Inez Vincent Cyclin A may not have any effect without coexpression of Cdk2. Perhaps this suggests that there is no endogenous Cdk-like activity in neurons that would be supported by cyclin A alone?

Vikram Khurana Inez, at least in the fly, expression of cyclins alone can quite potently activate the cell cycle, but activation of Cdk1 or Cdk2 alone do not.

Karl Herrup Vik, of the various Cdks and cyclins you tried, which way would you say the balance goes? More induce death or not?

Vikram Khurana Karl, do you think it is possible that what we are seeing in the AβPP mice are actual attempts to repair DNA via DNA replication, and that in the context of increased DNA damage/oxidative stress (which presumably do not occur in these models), apoptosis would be more widespread?

Karl Herrup Vik, I do not know. I do know that our work in the AβPP mice and in the ataxia telangiectasia (AT) mice (and, by the way, both human models of the mouse disease) indicates that cell cycle initiation may be necessary, but it is not sufficient for neuronal death. Something else has to happen. That is Mark's two-hit idea, maybe.

Jin-Jing Pei Vik, I feel it is strange that you did not see total tau change in your models. Have you tried other antibodies to total tau?

Vikram Khurana Jin-Jing, we have not tried non-C-terminal tau antibodies as yet.

Gabrielle Strobel Azad, you have a paper in Neuron this week about apoptotic signaling that is specific to neurons [16]. Do you see relevance of this work to the issue of cell cycle reactivation in neurodegeneration?

Azad Bonni Sure, I do. Pin1 acts downstream of Cdk1 (Cdc2) in the cell cycle. It would be interesting to look at Pin1 in neurological settings and, in particular, downstream of Cdc2 (such as the paper here).

Luc Buee Pin1 is not only a cell cycle protein. Its expression is also physiologically increased during neuronal differentiation.

Xiongwei Zhu Even in AD neurons it is hard to say whether cell cycle reentry leads to neuronal death. Since Inez clearly demonstrated Cdc2/cyclinB1 staining in lots of AD neurons, it is hard to believe that all these neurons will subsequently undergo apoptosis, which will deplete the brain within months [17]. Therefore, we proposed a two-hit hypothesis suggesting that both mitotic signaling and oxidative stress can exist individually and represent a single hit; only after the second hit do the neurons begin to die.

Inez Vincent But in the AβPP mutants, you already have two hits, that is, AβPP mutation and cell cycle activation. It would be great to figure out how to push this further.

Xiongwei Zhu Whether AβPP mutation is a hit independent of the cell cycle hit is unclear.

Greg Brewer Low ATP levels will induce death in the cell cycle. Has anyone looked at ATP levels in the context of these other genetic predispositions? Maybe excitotoxicity (AD stress) enters as a second hit.

Karl Herrup Greg, do you think that a metabolic stressor would push the neurons over the edge? What would you do to lower ATP levels?

Mark Smith Greg, I agree that this would be a likely "hit." Certainly we know that metabolic deficits are important in AD.

Jin-Jing Pei Hypoxia could be an approach.

Greg Brewer Karl, titrate in glutamate or a complex environment or a new animal in the cage.

Karl Herrup Vik, another question. Death and cycle are intermingled, but are they in the same cell? Did you double-label?

Vikram Khurana Karl, we tried double-labeling but unsuccessfully. Technically, the experiment did not work perfectly or else temporally, apoptosis and cell cycle activation may be separated.

Karl Herrup Vik, I think the latter may very well be the case. Remember, I said this was Wonderland.

Mark Smith Karl, please expand on Wonderland: Cell cycle does/does not cause cell death?

Karl Herrup Mark, cell cycling may be a necessary first step, but there is a lot going on, I think, once the neuron heads down that road.

Inez Vincent Referring to Karl's earlier point about replication, I do think it is absolutely necessary to demonstrate [DNA] replication when thinking about linking the cell cycle to pathology. Given that most other cell cycle regulators are not normally expressed in postmitotic neurons, their ectopic expression could cause multiple defects that may not necessarily cause replication. In this case, it would not be correct to deduce an involvement of the cell cycle. I have to agree with Karl that many in the field of neurobiology are currently redefining what apoptosis is in a neuron. I think we are going to realize that this represents a series of different mechanisms – caspase-dependent, independent, or cell cycle-dependent.

Gabrielle Strobel Inez, in light of what you just said, replication being a requirement for being sure one can invoke cell cycle, do you see offshoots from initial cell cycle protein upregulation to synaptic biology as well? I find that fascinating.

Inez Vincent Gabrielle, I have not concentrated on the synaptic end but am definitely becoming more interested.

Azad Bonni Inez, how does replication cause degeneration?

Inez Vincent I think probably in more than one way. The sudden activation of many different ki-

nases/phosphatases in the wrong environment is likely to cause devastation, especially in a cell that relies so heavily on signaling and cues. I had thought previously that the profound downstream effects – replication for one would also be detrimental – would be problematic, but now Karl has shown that neurons seem not to care as much about aneuploidy. On the other hand, cell cycle molecules seem to play a more direct role in neuronal apoptosis.

Azad Bonni I am not aware of DNA replication in cell death in most developmental situations that I know of (non-pathologic). However, this does not mean that the mechanisms of cell death are very different. For example, the proteins of the cell cycle (without inducing DNA replication) could engage the cell death machinery directly. What I am trying to say is that in degenerating neurons, perhaps we should look at direct links as well (in addition to trying to link DNA replication to the process).

Vikram Khurana Azad, I think in this context, Greene's recent paper demonstrating links between E2f and chromatin remodeling at the promoters of proapoptotic genes in the context of DNA damage is also very interesting [18].

Mark Smith With respect to apoptosis, this would be very, very rare in AD [19], but the cell cycle is common. Any thoughts?

Karl Herrup Mark, the difference is kinetic. Apoptosis is quick. Death by cycle is slow. At any one time that means there will be lots of the latter and little of the former.

Xiongwei Zhu Karl, is there any direct evidence suggesting how long death by cycle takes?

Karl Herrup Xiongwei, in mouse (the R1.40 AβPP mouse) it can be up to 2 years.

Xiongwei Zhu Karl, it is rather an inference since no death is demonstrated in the mouse model.

Karl Herrup Xiongwei, it is a lower limit. As there is no cell death, there is nothing to suggest that the mouse neurons could not remain in this state permanently. A chilling thought.

Vikram Khurana To Donna and Rachel, have there been any further developments on the PAK3 connection to ectopic cell cycle events in AD? Knockout PAK3 mice, I believe, exist and have substantial neural deficits. But I wonder if a PAK3 heterozygous background might be protective in AD/tauopathy models? I am interested that PAKs have recently been shown to activate the Aurora kinase, which may play into triggering late cell cycle events.

Rachael Neve Vik, I like your idea of PAK3 heterozygosity perhaps being protective. Our idea, though, is that the normal (probably synaptic plasticity) function of the AβPP/PAK3/AβPP-BP1 pathway is constitutively activated, and it is this constitutive activation of a normally beneficial pathway that sends the neurons into the cell cycle.

Inez Vincent Vik, I found it hard to tell where most of your cell cycle markers were localizing within neurons. Were they in the nucleus?

Vikram Khurana Inez, the cell cycle markers were found in the nucleus (PH3) or in both the nucleus and cytoplasm (PCNA).

Karl Herrup When we do modeling with embryonic neurons (or in developmental situations), I think they are in the same cells, but in the adult, all bets are off.

Vikram Khurana It is true. That is where I think Azad's work in cell culture systems may provide important molecular links. As Inez commented, cell cycle proteins can clearly have non-cell cycle function in neurons.

Greg Brewer Does anyone know about the signal to disassemble the cytoskeletal microtubules to get a pool of tubulin ready for mitosis/kinetichore microtubules?

Cathy Andorfer I think the microtubule instability may be the key. Different cell types may have different sensitivities and different responses.

Gabrielle Strobel Cathy, can you suggest molecular links connecting cell cycle to microtubule instability?

Vikram Khurana Cathy, it is interesting that altering microtubule stability in culture can actually lead to TOR-dependent apoptosis in cell culture models.

Cathy Andorfer Gabrielle, not exactly, but from a practical perspective, if a cell is preparing to divide,

there must be microtubule changes, and it has been shown that altered microtubule dynamics lead to apoptosis. There may be some tipping point, pathologically speaking, that sends some cell types into a death pathway and others into a paused cycling state, and perhaps still others to form tangles.

Jin-Jing Pei Activation of mTOR/S6K could cause tau phosphorylation directly, especially at Ser262. This might affect microtubule stability.

Vikram Khurana Jin-Jing, we do not show it in the paper, but TOR modulation also modulates our pseudophosphorylated E14 construct, suggesting TOR is not acting in our model through modulation of tau phosphorylation.

Jin-Jing Pei Vik, I am very much interested in the total tau level in your models. Are you going to check it again?

Vikram Khurana I can certainly do it. Which antibody would you recommend? We generally use C-tau and tau-5 for total levels.

Jin-Jing Pei Tau-5 or tau-2.

Vikram Khurana Jin-Jing, have you had troubles with C-tau in this regard?

Jin-Jing Pei No, it is better to check with antibody to full length, I think. Vik, after your experiments, do you think mTOR is a survival signal or a death signal?

Vikram Khurana Jin-Jing, blocking TOR blocks degeneration. I therefore argue for it being a death signal in our model.

Jin-Jing Pei Vik, in our experiments in different cell lines, mTOR/S6K activation is a survival signal.

Azad Bonni Jin-Jing, interestingly, Akt is widely considered to have pro-survival effects, but Harry Orr has shown that Akt induces degeneration in flies in a model of polyglutamine repeats [20].

Gabrielle Strobel Azad, does this point to differences between fly and other species, or different outcomes of Akt signaling depending on the particular pathway?

Azad Bonni Gabrielle, that is a very interesting point you are making. I think that the site in the protein they were studying (leading to 14-3-3 interactions) also occurred in mice. However, you are raising a good point.

Jin-Jing Pei Azad, yes. Akt is overactivated in tangle containing neurons in AD as well, and it can phosphorylate tau similar to p70S6K.

Vikram Khurana Azad, we have had mixed results with Akt in our model, possibly just the reagents we have or that multiple pro/anti-survival pathways are operating.

Gabrielle Strobel Jin-Jing, those neurons are still alive, presumably. Could Akt be compensatory?

Jin-Jing Pei Gabrielle, yes or no; I am not so sure yet.

Karl Herrup Vik, I remain intrigued by the similarities between Figs 3A and 3E. The data seem to say that the phosphorylation is nearly irrelevant. This flies in the face of the AD "lore." Of course, one missing control is the non-phosphorylatable tau. Did you try that?

Mark Smith Vik, I liked Figs 3A/E. . . did not fly in my face [21].

Vikram Khurana Karl, we show that expressing the pseudophosphorylated construct in the brain (Fig. 3, E14 graphs) causes 10 times more apoptosis in the brain. It is true that the effect in the eye is less dramatic, though.

Karl Herrup Vik, point taken. What is the difference with the eye? Are there secondary loss(es) in brain?

Vikram Khurana Karl, it could be timing. After the lens of the fly eye develops, degeneration of retinal neurons will not be visible from the outside but only histologically. Since we regularly score degeneration in the eye simply by inspection, we might be missing neurodegeneration that is going on underneath.

Karl Herrup Vik, have you tried alanine instead of glutamate substitutions in tau for their effects on neurotoxicity?

Vikram Khurana Karl, we have a paper in press with the alanine construct.

Cathy Andorfer Inez, Karl, I absolutely agree that death processes in neurons need much further explo-

ration. The death processes in the human tau mice were quite varied.

Luc Buee Cathy, how do you discriminate between cell cycle activation due to cell death and that due to neurogenesis in your mice?

Cathy Andorfer Luc, mostly via double-labeling confocal microscopy and BrdU incorporation. Cells that are newly synthesizing DNA will pick up the BrdU; if those cells are positive for mature markers (MAP1) and not positive for newborn markers like doublecortin (DCX), it is strong evidence that these are mature cells that are abnormally synthesizing.

Jin-Jing Pei Vik, I think the gap between mTOR and neurodegeneration is quite big, and worth exploring more in your models.

Filip Lim Perhaps the differentiation state is important in determining Akt/TOR or other kinase roles in survival or death. In our SHSY5Y models, if full differentiation is not accomplished, we observe death-promoting activity of factors which are non-toxic on the full differentiated cells, for example, tau.

Vikram Khurana Kiran, do you think Src mediates tau-induced cell cycle activation? Can you block cell cycle activation by blocking Src?

Kiran Bhaskar Vik, I did try double-labeling PCNA and tyr phosphorylated tau in a P301L mouse model and saw partial colocalization. I still need to work on that.

Mark Smith Quick last question: What do you think we need to do to truly put cell cycle front/center in AD field?

Vikram Khurana Mark, I think Cathy needs to block neurodegeneration and show behavioral recovery in a tauopathy mouse as the next step toward therapy.

Daniel Geschwind I would love to see some large studies trying to correlate these pathways with vulnerability, not just focusing on one pathway, but many.

Gabrielle Strobel We are nearing the end of the hour. Let me thank you all for coming and driving such a lively and spirited conversation.

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