Prionic Loops, Anti-Prions, and Dependence Receptors in Neurodegeneration

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1 Introduction

The 20th century has been dubbed the century of physics, and the 21st, the century of biology (Venter, 2004). Therefore, it is fitting that Stan's Nobel Prize was a fin de siècle award, presented as it was in 1997, close to the intersection of the two centuries, since his contribution spanned the two fields. Just as the original contributions to the nascent field of molecular biology sprang from the minds of physicists such as Schrödinger and Delbruck, searching for new physical principles but finding instead a new quantal genetics, the discovery of the prion involved physical, chemical, and biological studies ranging from radiation inactivation to Fourier transform infrared structural analyses to streamlined infectivity studies of genetically modified hosts, among many other creative and exacting approaches utilized by Stan and his co-workers.

I am grateful to Stan for allowing me to train as a postdoctoral fellow in his laboratory. I had read with fascination about scrapie and other "slow viruses" and "unconventional agents" as an undergraduate, and I was enthusiastic to train at UCSF both because of Stan's exciting research and because of the outstanding academic neurology program there. Stan had brought together scientists from complementary backgrounds such as genetics, cell biology, neuroscience, and physical chemistry, all focused on the prion, making remarkably rapid and insightful progress in a field that had been known for the career-compromising chronicity of its experiments. I learned from Stan the synergy arising from such an approach, and in 1998, when I was recruited to build the Buck Institute for Research on Aging, I employed a similar approach, bringing scientists not based on departmental boundaries but rather on complementary approaches to aging and age-related disease mechanisms. When the Institute opened its laboratories in 1999, Stan was generous enough to serve as the keynote speaker at our inaugural symposium.

If the 21st century is indeed the century of biology, then hopefully the current decade is the decade for the development of the first truly effective therapy for neurodegenerative disease. If and when such therapeutics are developed, it will be in no small part due to Stan's research and vision: after offering us the first view of at least some—if not

many—of these illnesses as prion-based diseases (Prusiner, 2012), he has established and driven a major effort in translation.

2 Neurodegeneration and programmed cell death (pcd)

Stan had discovered and coined the term prions in 1982, and when I established my own laboratory in 1989, I wanted to address two related questions: first, why do neurons degenerate in neurodegenerative diseases such as Creutzfeldt-Jakob disease and Alzheimer's disease? Second, is the neurodegenerative process mediated by developmentally-related, physiological signaling, or is it mediated by purely pathological, non-physiological processes? To address these questions, we needed a simple, rapidly iterative, genetically manipulable yet disease-relevant model, and unfortunately, no such model existed in 1989 (which made me envy the oncology researchers, who had well characterized cell culture phenotypes, readily transfectable cells, and remarkable in vitro-in vivo correlations in their model systems). Furthermore, there was resistance to developing and utilizing such a model: at neurodegenerative meetings in the early 1990s it was argued that any process that occurred rapidly in an in vitro system was unlikely to have much relevance for processes that occurred over years in vivo in chronic neurodegenerative conditions. It seemed like a rational argument at the time, but fortunately it turned out to be incorrect.

We initiated our laboratory efforts by evaluating the effects of antisense RNAs on glutamate toxicity in cerebellar granule neurons, in order to determine which gene products played critical roles in the process, but soon found that this system was not rapid enough or efficiently transfectable enough, given the technology available at the time. Therefore, we searched for alternative simple models of the neurodegenerative process. Eugene Johnson had published classic studies of neuronal programmed cell death induced by the withdrawal of trophic support from peripheral neurons in culture (Martin *et al.*, 1988), and these drew on the original concept of programmed cell death as described by Richard Lockshin in 1965 (Lockshin & Williams, 1965) and the description of apoptosis by Kerr (Kerr *et al.*, 1972). Johnson and his colleagues showed that gene expression was required for programmed cell death to occur in neurons following the withdrawal of trophic factors. The gene products mediating programmed cell death in simple systems such as C. elegans were beginning to be defined, and therefore we began to ask whether these same mediators affected models of developmental or degenerative neural cell death.

We were surprised to find that Bcl-2, which had been implicated in B-cell lymphoma development, had a far-reaching effect on cultured neural cells: whether the cells were insulted with oxidants, calcium ionophores, glucose withdrawal, trophic support withdrawal, or any of a number of other insults, Bcl-2 inhibited their apoptosis (Kane *et al.*,

1993; Mah *et al.*, 1993; Zhong *et al.*, 1993). Similarly, p35, a gene from baculovirus, also inhibited apoptosis induced by a wide range of insults, arguing for highly conserved mediators of the cellular response to a wide range of insults (Rabizadeh *et al.*, 1993a).

During the course of these studies, Fas was shown to be a death receptor, with its apoptosis induction requiring a 68-amino-acid death domain (DD) that is also present in members of the tumor necrosis factor receptor (TNFR) superfamily (Itoh & Nagata, 1993). This led to an interesting paradox: p75NTR, the common neurotrophin receptor, displays a DD and indeed was the founding member of the TNFR superfamily; however, p75NTR binds trophic factors [e. g., nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)], i. e., anti-apoptotic factors, rather than pro-apoptotic factors such as Fas ligand or tumor necrosis factor. To complicate the issue, the role of p75NTR in neurotrophin responses had been called into question, since its co-receptors, the tropomyosin receptor kinases (TrkA, TrkB, and TrkC), appeared to be capable of mediating the survival and differentiation effects of the associated neurotrophins. So what was the role of p75NTR?

Expression of p75NTR in neural cells led to an effect that was the mirror image of the effect of Fas: rather than mediating apoptosis upon ligand binding, the expression of p75NTR induced apoptosis *unless* a neurotrophin ligand was supplied. Thus the expression of p75NTR conferred a state of neurotrophin dependence on its expressing cells (Rabizadeh *et al.*, 1993a; Rabizadeh & Bredesen, 1994). We subsequently identified similar effects of other trophic factor receptors e. g., RET [rearranged during transfection (Bordeaux *et al.*, 2000)], DCC [deleted in colorectal cancer (Mehlen *et al.*, 1998)], and Unc5H1-3 [uncoordinated homologues 1-3 (Llambi *et al.*, 2001)], and therefore dubbed these dependence receptors. To date, over 20 such receptors have been described (Mehlen & Bredesen, 2011). These receptors are essentially ligand-modulated molecular switches that share a number of functional and mechanistic features: (1) they induce pcd in the absence of the trophic support of their respective ligands, but typically inhibit apoptosis in the presence of their trophic ligands (Rabizadeh *et al.*, 1993b; Mehlen *et al.*, 1998); (2) they complex with specific caspases, and participate in caspase activation and amplification (Lu *et al.*, 2003a); (3) their signaling features proteolytic cleavage of the receptors themselves, typically by caspases at a single or double intracytoplasmic site (Mehlen *et al.*, 1998; Bordeaux *et al.*, 2000); (4) point mutation of the caspase site in each receptor prevents the dependence effect (Mehlen *et al.*, 1998; Ellerby *et al.*, 1999a; Ellerby *et al.*, 1999b); (5) caspase-derived fragments of these receptors exhibit pro-apoptotic activity (Lu *et al.*, 2000); (6) these receptors may mediate sub-apoptotic events such as neurite retraction and somal atrophy, as well as apoptosis (Yeo *et al.*, 1997); (7) mutations in these receptors are associated with neoplasms such as breast cancer and neuroblastoma (Thiebault

et al., 2003; Delloye-Bourgeois *et al.*, 2009), developmental neuronal abnormalities such as Hirschprung disease (Bordeaux *et al.*, 2000), or neurodegenerative disorders such as Kennedy's disease (Ellerby *et al.*, 1999b). Interestingly, the well-described phenomenon of spontaneous regression of type IV-S neuroblastoma has been linked to dependence receptor expression (Delloye-Bourgeois *et al.*, 2009). It should be added that recent work from the laboratory of Yves Barde has shown that TrkA and TrkC (but not TrkB) are also capable of mediating neurotrophin dependence, by a mechanism that requires p75NTR (Nikoletopoulou *et al.*, 2010).

Given that the expression of p75NTR is highly restricted in the central nervous system, with the main expressors being the basal forebrain cholinergic neurons; and given that these same neurons are preferentially affected in Alzheimer's disease; the question of whether p75NTR is a mediator of Alzheimer-related pathophysiology arose. We found that the expression of p75NTR sensitizes cells to the toxic effects of amyloid-beta (Aβ) peptide (Rabizadeh *et al.*, 1994), and subsequently it was shown that Aβ interacts directly with p75NTR (Yaar *et al.*, 1997). These findings led to the development by Steve Massa and Frank Longo of a novel AD therapeutic, currently in pre-clinical testing, that binds p75NTR (Yang *et al.*, 2008).

The finding that p75NTR is a mediator of death following neurotrophin withdrawal led to the obvious question of whether, as an alternative to simple trophic factor withdrawal, there might be an as-yet-unidentified "anti-trophin" that would trigger or enhance such a response. Such a molecule would be predicted to interact with one or more dependence receptors, reduce trophic signaling, and mimic the signaling induced by trophic factor withdrawal. It has now become clear that Aβ peptides fulfill the appropriate criteria to be designated anti-trophins, and we have suggested that this activity represents one of the physiological functions of these peptides (Bredesen, 2009). For example, Aβ binds to the insulin receptor and inhibits kinase signaling (Townsend *et al.*, 2007), binds p75NTR and activates cell death signaling (Yaar *et al.*, 1997), and activates cell death signaling following binding to another dependence receptor—APP itself (Lu *et al.*, 2003a).

During the course of our studies on trophic factor withdrawal-induced programmed cell death, the question came up, during a conversation with Dave Borchelt, whether human familial neurodegenerative disease-associated mutations would have an effect on apoptotic paradigms. In collaborative studies with Dave, we found that, whereas the expression of wild type copper/zinc superoxide dismutase (CuZnSOD) had an inhibitory effect on serum withdrawal-induced apoptosis in neural cells, mutations associated with familial amyotrophic lateral sclerosis (FALS) had a clearly pro-apoptotic effect (Rabizadeh *et al.*, 1995). This was the first example of what has turned out to be a general effect: virtually all neurodegeneration-associated mutants, when expressed in neural cells, increase

the probability that the cells will undergo apoptosis (Yamatsuji *et al.*, 1996; Martindale *et al.*, 1998; Tanaka *et al.*, 2001).

Our studies on the apoptotic effects of neurodegeneration-associated mutants and those on dependence receptors intersected when it became clear that APP displays several features common to dependence receptors, such as a predicted caspase site (D664) and the ability to generate pro-apoptotic peptides. We found that APP is indeed cleaved by caspases at D664, and that, just as for other dependence receptors, mutation of the caspase site prevents the generation of a pro-apoptotic fragment—in this case, APP-C31 (Lu *et al.*, 2000). This provided support for the notion that APP functions as a dependence receptor, and furthermore, APP appears to be an integrating dependence receptor, since the withdrawal of different types of trophic support (as opposed to being restricted to a single trophic factor) leads to APP-mediated caspase activation (Nikolaev *et al.*, 2009). These studies left unanswered the question of whether APP itself binds a trophic ligand. However, subsequent studies in collaboration with the laboratory of Patrick Mehlen disclosed netrin-1, an axon guidance and trophic factor, as a ligand for APP (Lourenco *et al.*, 2009).

The finding that the proteolysis of APP may produce two different pro-apoptotic fragments—Aβ and C31—raised the question of what role, if any, C31 may play in Alzheimer's disease pathogenesis. Therefore, we created transgenic mice expressing APP with Swedish and Indiana familial AD mutations, with an additional D->A mutation at the caspase cleavage site (D664A), matched for genetic background and expression level of APP with PDAPP mice, then evaluated these for features of the AD phenotype. The D664A mutation prevented the electrophysiological abnormalities, the dentate gyral atrophy, the synaptic loss, and the spatial memory abnormalities measured by the Morris water maze (Galvan *et al.*, 2006; Saganich *et al.*, 2006), without affecting Aβ production or plaque formation. These results ran counter to prevailing models of AD as a disease due to chemical and physical effects of Aβ peptides, such as reactive oxygen species generation, detergent-like effects, or metal binding, and suggested instead that APP-mediated signaling plays a key role, at least in the transgenic mouse model based on familial AD.

Following our report on the generation of C31 from APP, two other groups reported additional pro-apoptotic peptides derived from APP: Jcasp (Bertrand *et al.*, 2001) and N-APP, the latter of which was demonstrated to bind to DR6 and induce caspase-6 and neurite retraction (Nikolaev *et al.*, 2009). Thus APP may give rise to four pro-apoptotic peptides, when processed at the β (and N-APP), γ, and caspase sites; conversely, when cleaved at the α site, APP gives rise to two peptides—sAPPα and αCTF—that display opposing effects, inhibiting apoptosis and supporting neurite extension rather than retraction. Therefore, APP appears to be set up to function as a "plasticity switch," signaling either neurite extension, synaptic maintenance, and caspase inhibition, or neurite retrac-

tion, synaptic reorganization, and caspase activation (Bredesen, 2009). If that is the case, what throws the switch and determines the APP-mediated signaling pathway? Ligand engagement is one determining factor: when netrin-1 binds APP, the sAPPα pathway is increased (Lourenco *et al.*, 2009), whereas when Aβ (which competes with netrin-1) interacts with APP, the neurite-retractive, pro-apoptotic pathway is increased (Lu *et al.*, 2003b). Thus the Aβ-APP interaction begets additional Aβ, and therefore forms a prionic loop. In contrast, netrin-1 functions as an endogenous anti-prion, since it inhibits this loop and reduces Aβ (Bredesen, 2009; Lourenco *et al.*, 2009). Furthermore, multiple other receptors may interact with APP and affect this switching — e. g., p75NTR interacts with APP and increases the Aβ processing, thus being prionogenic (Fombonne *et al.*, 2009).

These findings suggested a new model of Alzheimer's disease as a trophic vs. antitrophic signaling imbalance (Fig. 1), mediated by dependence receptors and amplified by prionic loops. This model, if correct, would explain a number of the findings that are unexplained by current theories, and would also suggest novel approaches to therapy.

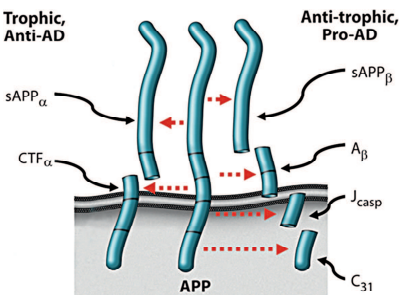

Fig. 1: Alternative cleavage of APP to produce four peptides that mediate neurite retraction, caspase activation, and synaptic reorganization; or two peptides that display opposing effects, inhibiting apoptosis, supporting neurite extension and synaptic maintenance.

3 The dependence receptor theory of Alzheimer's disease: Therapeutic implications

Any accurate theory of AD should address the many epidemiological, genetic, and neurochemical findings associated with AD, including the following:

- The AD11 mouse.
- The α7 paradox.
- Lack of successful therapeutic development to date.
- The remarkable diversity of risk factors for AD.
- The high prevalence of AD in the elderly.
- The mechanism(s) by which ApoE4 increases risk for AD.

- The physiological role(s) of Aβ peptides.
- The anatomic pattern of spread of AD pathology.
- The association of plastic brain regions with AD pathology.
- Why some people (and transgenic mice) collect large amounts of Ab peptide without displaying symptoms of AD.
- The relationship between Aβ and tau pathology.

For example, the AD11 mouse derived by Cattaneo and his colleagues (Capsoni *et al.*, 2011) develops plaques and tangles associated with a chronic reduction in NGF; by what mechanism? Why is AD risk increased by such disparate factors as the ApoE ε4 allele, early oophorectomy, metabolic syndrome, head trauma, and hyperhomocysteinemia? Why have therapeutic attempts to date been so unsuccessful?

Consider the most basic and simplistic view of AD as an imbalance in connectivity, analogous to the imbalance in proliferation and survival that occurs in neoplasia. Organismal development at its most basic requires four processes following zygote formation: proliferation, differentiation, migration (of both cell bodies and processes), and integration (Fig. 2). For lower species like C. elegans, these developmental processes are terminal: C. elegans survive approximately three weeks, with little in the way of regenerative proliferation, migration, or integration. However, for higher species like H. sapiens, these same processes are employed for repair and regeneration, so that, for example, humans may live 100 years instead of the three weeks afforded C. elegans. However, in so doing, a life-long requirement for balance in these processes is created. This is critical for the avoidance of disease, since it has turned out that the physiological control of each of these basic processes features positive, i. e., anti-homeostatic, feedback.

A great deal has been written about the result of imbalances in proliferation and survival vs. turnover (programmed cell death). Such an imbalance may occur due to genetics or exposure to carcinogens, for example, and result in oncogenesis. A multitude of initiators may play into this mechanism, but their common feature is an imbalance toward an increase in the oncogene to tumor suppressor gene function. The positive feedback in this case is provided by the Darwinian selection for cells with enhanced proliferation and/or survival. This selection process is dynamic, resulting in the continued progression in the neoplastic phenotype.

Our results on the trophic-antitrophic balance that affects APP processing suggest that an analogous mechanism operates in the balance of migration and integration. Just as oncogenes and tumor suppressor genes balance proliferation and survival with turnover, gene products controlling processes involved in plasticity—specifically, neurite extension vs. retraction, synaptic maintenance vs. reorganization, caspase inhibition vs. activation, synaptic efficacy vs. inhibition, and related processes—create balance in process migra-

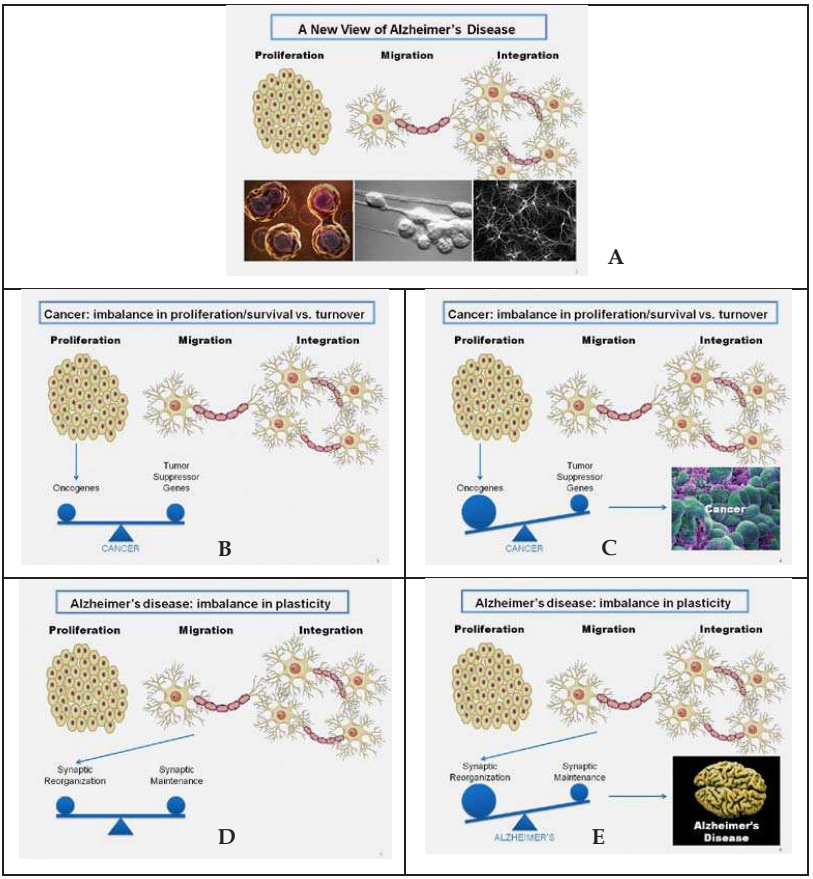

Fig. 2: (A) The four basic developmental processes of proliferation, differentiation, migration, and integration are utilized by higher organisms post-developmentally for repair and regeneration, creating a life-long need for balance in these processes. (B, C) Imbalance in proliferation and survival vs. programmed cell death, typically due to somatic or germ-line mutations, may result in cancer, and amplification of the initial abnormality is effected by Darwinian selection for cells that proliferate and survive at a rate greater than normal cells. (D, E) Imbalance in migration and integration, processes critically involved in plasticity, may result in Alzheimer's disease. This imbalance may be induced by genetic or age-associated reductions in the trophic-antitrophic balance, which may be associated with numerous risk factors such as hormonal, traumatic, metabolic, or other abnormalities. The amplification in this case, in contrast to the cellular-level amplification that occurs in cancer, is at the molecular species level, through prionic loops such as that created by the interaction of Ab with APP. This amplification raises the question of whether rare somatic mutations may in some cases initiate Alzheimer's disease.

tion and integration. APP processing and signaling have been implicated in all of these biochemical mechanisms. Interestingly, whereas the positive feedback in oncogenesis occurs at the cellular level, the positive feedback in the plasticity imbalance occurs at the molecular species level, i. e., it is mediated by prionic loops (Bredesen, 2009; Corset, in

prep.). Furthermore, not only is the disease-mediating signaling at least partly diffusible, but also, as for oncogenesis, there is a phenotypic progression, involving upstream mediators such as Aβ-APP and downstream mediators such as tau and WAVE1.

If Alzheimer's disease does result from an imbalance in migration and integration, initiated by a reduction in the trophic-antitrophic balance, mediated by dependence receptors such as APP, and amplified by prionic loops such as that formed by Aβ and APP, this raises the question of the origin of prionic loops. There are two fundamentally distinct types of feedback in biological systems: homeostatic and anti-homeostatic. In systems featuring a single goal outcome and no requirement for amplification, homeostatic feedback occurs, providing systems stability. An example is the preservation of serum pH at 7.4. However, in systems requiring amplification and featuring multi-goal outcomes, i. e., molecular switching systems, anti-homeostatic signaling occurs, providing metastability. An example is the blood clotting system, in which a series of serine proteases amplifies its own activity, resulting in a transient change in structure (the thrombus) that is degraded proteolytically over time, resetting the switch. An analogous event occurs with Aβ production and oligomerization, resulting in a transient alteration in synaptic structure and function that, instead of impeding blood flow, impedes synaptic flow. Such systems are by definition prionic.

Thus we suggest that Alzheimer's disease is fundamentally similar to the other chronic illnesses of aging, such as osteoporosis, neoplasia, and atherosclerosis. In the case of AD, all of the remarkably disparate risk factors identified share the common feature of reducing the trophic-antitrophic ratio. Therefore, therapeutic strategies to restore the balance in the trophic-antitrophic signaling network are of potential interest.

In order to evaluate this possibility, we screened chemical libraries for molecules that shift the balance of APP processing away from the production of the four neurite-retractive peptides and toward the production of the two neurite-extending peptides. One of the molecules identified in this screen was disulfiram, an inhibitor of aldehyde dehydrogenase. Surface plasmon resonance studies showed that disulfiram interacts directly with APP, with an affinity of approximately 100nM. Furthermore, structural studies, using small-angle X-ray scattering (SAXS) and fluorescence spectroscopy, showed that disulfiram's effects on APP are distinct from the effects of Aβ on APP. In the absence of ligand, APP forms a compact homodimer, with a major region of intermolecular interaction in the Aβ region and a minor region of interaction in the RERMS domain (Libeu *et al.*, 2011). Aβ monomers and dimers interact with APP homodimers, split the homodimers (producing heteromultimers of APP-Aβ), and "pop the top," opening up the compact structure of the E1 and E2 domains (Libeu *et al.*, 2011). In contrast, Aβ oligomers interact with APP homodimers and stabilize the dimers, inhibiting monomer formation, and also

opening up the compact structure. This distinction is compatible with previous findings that Aβ oligomers are toxic, whereas Aβ monomers are neuroprotective (Giuffrida *et al.*, 2009), as is sAPPα, which also prevents APP dimerization (Gralle *et al.*, 2009). Disulfiram monomerizes the APP homodimers as do Aβ monomers and dimers, but does not alter the compact structure (Libeu *et al.*, 2012). Both with purified extracellular APP (eAPP) and in cells, disulfiram increases the sAPPα to sAPPβ cleavage ratio. Therefore, it is possible that APP-monomerizing drugs such as disulfiram may represent one approach to increasing the ratio of APP-mediated trophic to antitrophic signaling.

Unfortunately, disulfiram showed poor brain penetration, and therefore additional candidates with similar properties, but better blood-brain barrier penetration, were sought. We have identified such candidates, and shown that they improve memory and biomarkers in the PDAPP transgenic mouse model of AD. The most promising of the candidates is set to advance to clinical trial in late 2012.

We have also taken a complementary approach, in order to enhance the effects of the "switching" drugs such as disulfiram. Several groups have identified BACE inhibitors, but since BACE cleaves several substrates other than APP, it would be optimal to identify APP-specific BACE inhibitors (ASBIs) rather than non-specific BACE inhibitors. There is a precedent for this approach in the development of molecular clamps. In our screens, we have identified molecules that interact directly with APP instead of BACE, inhibiting the cleavage of APP, but not other substrates, by BACE.

In summary, our studies of neural cell death in simple model systems have led to a first clinical trial with a novel therapeutic approach to Alzheimer's disease, and none of this would have been possible without the training provided by Stan and his laboratory members. Thank you, Stan, for giving me the opportunity to learn in your remarkable laboratory.

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