A Novel Approach for Preventing Neurological Disease Associated with Blood-Brain Barrier Pathology

The Problem

The strategy for treating disease has not changed markedly since the rise of modern pharmaceuticals in the past 50-100 years. The vast majority of drugs have been and continue to be disease modifying agents. They treat symptoms but they do not cure, and so they must be taken chronically. As they are merely suppressing symptoms, they may be only partially effective and can also carry strong side effects, leaving the patient in a limbo state: not quite sick, not quite healthy, constantly medicated to stave off a decline into full-blown illness. The costs of chronic treatment, to the patient and to our medical system, are enormous.

Consider, for example, the two diseases that we are first targeting in our novel therapeutic approach: age-related dementia and post-traumatic epilepsy (PTE). For both, there is currently no diagnostic method to predict who will acquire the disease. We know that certain populations are at risk. If you are at risk, you may get it and you may not. We simply wait until severe, late-stage symptoms appear, and then do what we can to manage them with a variety of drugs.

In contrast to slow changes in clinical approaches, fundamental biological research has advanced at a startling pace. Compared to 50 years ago, we can now peer into the detailed molecular mechanisms that cause disease, and in doing so understand that many diseases don’t appear at random, but rather are triggered by previous life events which set in motion biological changes that only later manifest as pathology. For example, for the past 10 years we have been studying a variety of different diseases that cause acute damage to the brain, such as traumatic injury (TBI), stroke, and brain tumors. While each of these diseases are quite different in the immediate treatment, the long-term outcomes for patients are remarkably similar: regardless of the type of brain injury, patients show a non-specific set of later pathology, ranging from mild cognitive decline and neurodegeneration to, in the most severe cases, appearance of chronic epilepsy\(^1\)\(^-\)\(^6\). These pathological endpoints appear after the patient has fully recovered from the initial injury, weeks to months or even years later\(^7\)\(^-\)\(^10\). Patient outcomes in traumatic brain injury (for example after a car accident) illustrate this point effectively. Each year there are approximately 2 million cases of traumatic head injury, and 10-40% of these patients later go on to develop epilepsy, while an even larger number show cognitive
impairment\textsuperscript{11,12}. Yet despite the clear link between head injury and epilepsy, there is no therapeutic approach to predict or prevent post-traumatic disease: no diagnostics to predict which post-injury patients are at risk, no drugs to prevent disease progression. Rather, post-traumatic epilepsy patients are only diagnosed once chronic seizures appear, and then are placed on anti-epileptic drugs which often fail to control seizures and impose strong cognitive side effects\textsuperscript{13}.

Our approach to this problem is completely different. Through sustained research, we have identified the common biological mechanisms that cause the brain to become reorganized and pathological after injury. By understanding these earliest stages of disease progression, we developed new therapeutics to target the pathways that induce pathology, thereby preventing post-injury changes. In other words, our approach is to prevent brain pathology before it starts, rather than chronically treating symptoms after disease has already progressed to a late, irrevocable stage (Figure 1).

\textbf{Figure 1.} We identified a common pathological pathway shared across a variety of brain insults; these injuries cause disruption of the blood-brain barrier, inducing an injury response that reorganizes the brain resulting in neurodegeneration, cognitive dysfunction, and epilepsy. As patients experience these injuries, they cause a delayed appearance of subsequent illness that is only treated once endpoint symptoms appear. Our strategy is to treat the root cause of disease, thus preventing the pathology before it starts.
Our Solution: Preventing Diseases Caused by Pathology of the Blood-Brain Barrier

Our story begins with trying to understand the common elements across different types of brain injuries: what changes induced by injury could subsequently lead to the pathological outcomes seen in the clinic? We quickly homed in on the blood-brain barrier (BBB), a specialized lining of cells that wrap the vasculature in the brain, serving as a filtering protective layer that keeps the brain’s environment separate from the blood circulating through the rest of the body. The unique environment maintained by the BBB is critical to maintaining healthy functions of neurons, yet it is disrupted in every major type of brain insult, which in turn allows components of the blood to leak into the brain and perturb its normal functions.

In our first phase of research, we showed that BBB disruption is the key cause of pathological changes resulting from head injury, and described the mechanisms that induce pathology. Firstly, we showed that experimentally disrupting the BBB in rodents, without causing any other injury, leads to the later development of pathology, including changes in neural function and appearance of epilepsy14,15,16. Secondly, we isolated the key molecule from the blood that induces the changes, and characterized the molecular mechanisms causing disease. We found that albumin, the most abundant protein in the blood, activates an inflammatory signaling response when it enters the brain (after BBB disruption). This occurs as a generalized injury response: once BBB is compromised, albumin leaks into the brain and binds to the transforming growth factor beta receptor (TGFβR), activating this master signaling pathway that regulates inflammation and injury-response12,15 (Figure 2: Appendix 1-2). The TGFβR is specifically activated on astrocytes, a type of cell in the brain that serves as an overall sensor and regulator of the brain environment. In turn, astrocytes become reactive, and secrete a variety of molecules via the TGFβ pathway that cause pathological reorganization of the brain and changes in neural function15. This albumin-induced inflammatory signaling in turn causes a range of pathological outcomes (Appendix 4): neurodegeneration and cognitive dysfunction, including neural cell death, reduction in cortical volume, and decrease in brain function15, and increases in neural excitability (reduced potassium buffering and changes in proteins regulating excitatory neurotransmitter release)15,16,17 and excitatory connectivity (increased synaptogenesis and aberrant neurogenesis in the hippocampus; Appendix 5)15,18,19. In the most severe cases, a subset of these animals developed chronic epilepsy, mirroring the human clinical condition of PTE20. Importantly, because this was a novel role for albumin in causing pathological activation of TGFβ signaling, we also characterized the specificity of this mechanism. We showed that albumin directly binds the TGFβR, and that blocking this binding prevents albumin uptake into astrocytes and initiation of pathological signaling15,16,20. We also used lipid-free and recombinant versions of albumin to show that albumin alone causes the pathology, while other control proteins of similar weight showed no pathological effects. Finally, we used microarray to compare the entire range of pathological signaling outputs that are caused albumin, and found that they were the same as the signaling cascade caused by BBB disruption or by direct activation of the TGFβ pathway by its canonical ligand, TGFβ15. Thus, activation of the TGFβ pathway by any method – by BBB disruption, by treatment with any form of albumin, or simply with TGFβ1 itself – causes the same pathological outcomes, validating the primacy of this pathway in BBB-initiated pathology.
After identifying the TGFβ pathway as the key cause of pathology following BBB disruption, we then sought to test the efficacy of this target to prevent pathological signaling and onset of disease after injury. Initially we used a variety of TGFβ blockers that were readily available in the experimental laboratory setting, including blocking antibodies and small molecule TGFβR inhibitors such as S8-431542 and S1N-2511. Subsequently, as we started to translate our research towards the clinic, we developed and tested drugs that have strong clinical properties that would allow for administration in patients. Our current lead molecule is IPW-5371, which we are developing with our partner chemist Dr. Barry Hart (Innovation Pathways). IPW is a novel small molecular inhibitor of the TGFβR kinase that has excellent clinical properties: it is orally bioavailable, readily crosses into the brain, and has a long half-life (present at effective concentrations for about 24 hours after a single dose in mouse), making it an ideal candidate for practical, clinical use (Figure 3). We have filed a disclosure for use of IPW in treating brain pathology, via UC Berkeley’s IP office (IPRA); under standard UC agreements, once awarded this patent will be licensed to us for our exclusive use. As another therapeutic candidate, we also have a patent filed for new use of losartan, a generic angiotensin II receptor antagonist now in the public domain, for treating brain pathology after BBB disruption. We selected losartan not based on its primary angiotensin activity, but based on its off-target effects in suppressing TGFβ pathway signaling21. Our rational was to identify an already FDA-approved drug targeting TGFβ signaling, with the potential to be quickly and cheaply brought to market under new use.

In all of these different approaches targeting the TGFβR, we found that blocking activation of the TGFβ signaling pathway effectively prevents all stages of the progression of disease pathology. This includes preventing the initial pathological activation of astrocytes, preventing the systemic induction of the inflammatory TGFβ pathway that causes pathological neurodegeneration and reorganization of the brain, and in turn preventing subsequent symptoms of disease progression including aberrant connectivity and increased neural excitability4,8,11,20 (Appendix 1-2).

Together, our data shows that targeting BBB pathology and associated activation of the TGFβ pathway as the earliest, causal event precipitating disease is a highly effective strategy; by blocking initial inflammatory signaling, we are able to completely prevent the ensuing chain of events leading to severe pathology in the brain. Because disruption of BBB is so widespread across many different neurological insults, our findings open up the potential for a completely new, preventative therapeutic approach with broad application across different diseases. Initially, we are focusing on targeted entry into two markets that have high incidence and are lacking effective treatments: post-traumatic epilepsy and age-related dementia.
Our Markets

Post-traumatic Epilepsy: Preventing pathology and chronic epilepsy after head injury

Our data shows that blocking albumin induction of the TGFβ pathway can prevent the pathological signaling changes leading neural hyperexcitability and epilepsy after head injury. To demonstrate the proof-of-principle of this strategy, we tested our therapeutics in three different models of brain injury in rodents, and followed the outcomes with long-term continuous electrophysiological recordings, electrocorticography (ECoG) of brain activity to detect epileptogenesis and seizures in real-time. Firstly, we infused albumin directly into the brain ventricles via intracerebroventricular (ICV) osmotic pumps, while also co-delivering a TGFβR inhibitor (SIN) directly into the ventricles. This shows the fundamental, disease-causing effects that albumin has on the brain, without any other injury, and the effectiveness of blocking the target pathway. Secondly, we opened a surgical window to the cortical surface and perfused albumin onto the surface of the brain, and co-treated with losartan during the surgery. This mimics a superficial injury, such as occurs in head trauma. Finally, we tested a model designed to recapitulate the sequence of events that would happen with an actual clinical patient. Rather than surgically administering albumin, we opened the BBB to closely mimic the pathology that occurs in head injury; we then acutely treated with oral administration of losartan for three weeks via the drinking water, as would occur in a theoretical patient taking our preventative therapeutics in the acute phase after injury, during recovery. After three weeks, drug administration was halted, and the long-term outcomes were followed. In each of these injury models, most of the subjects developed epilepsy after injury if they were not treated with our therapeutics;

Figure 4. Blocking activation of inflammatory TGFβ signaling prevents epilepsy in three different models. Continuous recordings detecting seizures are shown as heat maps (each line is one animal; increasing number of seizures on a given day is shown as red on the heat map and blue is no seizures). In the bottom row, data for each experiment is quantified as % of animals with epilepsy and average number of seizures. Note that control animals show a limited number of seizures due to sham surgeries (i.e. performing surgery but without albumin delivery).
in contrast, preventative intervention with our therapeutics, at the time of initial injury, completely prevented the onset of epileptogenesis and the appearance of chronic seizures\textsuperscript{18,20} (Figure 4).

\textit{Market analysis:} Epilepsy affects 2.5 million people in the US with an estimated annual cost of $15.5 billion in healthcare\textsuperscript{22}. The market for pharmaceutical treatment of epilepsy generated $12 billion in 2008, and is estimated to increase by 30\% by 2016\textsuperscript{23}. In cases where the etiology is known, the largest cause of symptomatic epilepsy is traumatic brain injury (TBI). In the US, there are 1.5 million TBIs resulting in ER care each year, making TBI the largest cause of death and disability in patients 1 to 44 years old. The relevant incidence of TBI is likely much higher since many patients do not seek ER treatment (e.g. concussion). 10-40\% of TBI cases result in epilepsy, and 5.3 million patients suffer other disabilities from TBI including cognitive dysfunction\textsuperscript{42}. Because our approach is highly disruptive, offering a preventative treatment for the otherwise incurable, chronic disease of PTE, we believe we can quickly capture market dominance if we succeed in clinical trials. Our diagnostics are feasible for screening every TBI patient in facilities that have the basic MRI infrastructure, giving clinicians the potential to know when to prescribe our therapeutics. Furthermore, even in settings without diagnostic capability, the acute and preventative nature of our treatment makes it feasible for prophylactic use after TBI to prevent the potentially severe, life-long debilitation of post-traumatic epilepsy. Furthermore, BBB disruption, the key step in PTE, is also a common event in stroke and brain tumors, each of which can also lead to epilepsy. The incidence for such markets in the US alone (from CDC reports, cases per 100,000 people) is 327.9 for stroke, and 6.4 for brain tumors. Success in our therapeutics in post-traumatic epilepsy would provide a route for future entry into these other markets (Appendix 3).

\textit{Age-related dementia: treating the BBB breakdown that occurs in aging to prevent and reverse cognitive decline}

While disruption of the BBB can occur acutely following the physical damage of head injury, a more widespread, indeed nearly universal, cause of BBB breakdown is decline during aging. Like many body systems that fall into disrepair during senescence, the integrity of the BBB declines during aging, causing widespread BBB “leakiness” that has been validated in human imaging studies, specifically in the hippocampus\textsuperscript{23}. Our research shows that activation of TGF\textbeta{} signaling after BBB disruption causes a range of pathological outcomes, from neurodegeneration and mild cognitive impairment to severe epilepsy, and these symptoms almost exactly match the ranges of pathology seen in aging. Indeed, while dementia and cognitive impairment are the most well-known aspects of neurological decline in aging, it is less well known that the highest incidence of epilepsy occurs in the elderly population. Thus, we reasoned that BBB disruption and albumin-induced TGF\textbeta{} signaling may be a major contributor to the range of neurodegenerative symptoms seen in aging.
To investigate this, we analyzed the status of BBB in otherwise healthy, normally aging mice. We found aged mice show chronic leakiness of the BBB with high amounts of albumin present in the brain, whereas young adult mice always have a healthy, intact BBB. We further found that the pathological activation of TGFβ signaling in these aged mice was nearly identical to the pathology that we see in young mice with all the hallmarks astrocytic activation and inflammatory signaling that trigger subsequent disease (Appendix 6). Critically, we found that treating mice with a daily dose of IPW can reverse reactive gliosis and inflammatory signaling. Similarly, we obtained post-mortem sections from aged human

![24 month old mouse](Image) ![77 year old human](Image)

BBB pathology is present in aging (but not young) mice and humans, including albumin extravasation and inflammatory signaling

![Young vs old mouse](Image) ![Albumin treated mouse](Image)

ECoG recordings from aged mice show increase in power of delta and theta bands, similar to dysfunction seen in young mice treated with albumin

![Novel object memory](Image) ![Mortality from seizures](Image)

Treating aged mice with IPW improves memory performance. IPW also improves resistance to induced seizures.

**Figure 5.** Loss of BBB barrier integrity in aging is associated with pathological inflammation and aberrant neural activity. Treatment with IPW, our lead therapeutic, reverses cognitive decline in the novel object memory task and reduces vulnerability to seizures in aged mice.

brains, and found the same evidence of albumin and activation of inflammatory TGFβ signaling (Figure 5). We also searched for early signs of neural dysfunction by performing continuous telemetric ECoG recordings from the brains of aging mice, and found that aging mice show aberrant appearance of slow wave rhythms during normal brain function, which are absent in young mice; the same shifts in slow wave power in brain activity were seen in young mice when after treatment with albumin to induce inflammatory signaling (Figure 5; Appendix 7). Together, these data suggest that BBB breakdown and inflammatory TGFβ signaling may be a major cause age-related neurological pathology. To test this in a clinically relevant model, we treated mice with daily doses of IPW, our lead TGFβR-blocking drug, and assessed a range of disease outcomes from cognitive impairment to seizure vulnerability. Mice given one week of daily IPW showed significant improvement in memory function. Furthermore, one week of IPW treatment drastically reduced the vulnerability of aged mice to induced seizures, decreasing seizure mortality at the given dose and decreasing the severity of seizures when they occurred (Figure 5).
Market analysis: Cognitive impairment affects 16 million Americans, and the number of people 65 and over diagnosed with dementia is expected to rise by 6.1 million by 2050\(^7\). Cognitive impairment is costly, causing hospital stays that are three times more frequent than for other diseases\(^2\). Dementia alone is estimated to be the third most expensive disease to treat in the United States. Medicaid nursing facility spending for individuals with Alzheimer’s disease is estimated at $47.6 billion\(^2\). Unpaid care by family members is also a huge expense, estimated at 12.5 billion hours provided for a value of $144 billion\(^2\).

Our approach: Using Diagnostics to Guide Preventative Treatment

Bringing our preventative approach into clinical use entails a number of challenges, one of which is improving the diagnostic capability and understanding of who is at risk for acquiring cognitive decline and epilepsy. Considering our target markets of post-traumatic epilepsy and dementia in aging, there is currently no way to diagnose risk or predict disease prior to onset of symptoms. In identifying BBB dysfunction as a very early, initial event leading to later pathology, we are seeking to not only develop early preventative therapeutics, but also use BBB status as a diagnostic to predict disease onset; in other words, our research suggests that disruption of BBB should be a powerful, and previously unknown, predictor of future disease progression.

![Figure 6. Our diagnostic imaging reveals breakdown of BBB in healthy American football players that suffer “mild” repeated head hits below the level of clinical concussion.](image)

Seeking to realize the potential of such diagnostics, we developed a new imaging approach using contrast-enhanced MRI, combined with our proprietary software analysis, to detect disruption of the BBB\(^27\) (Appendix 8). In this approach, the injected contrast agent is delivered IV and circulates through the blood. Normally, the contrast agent is excluded from the brain by the BBB, but when BBB is disrupted it leaks into the brain, where it is detected as an increase in signal intensity in MRI.

As proof-of-principle, we have tested this method in both of our target markets. In traumatic brain injury, we performed diagnostic imaging in American football players. These subjects represent a “mild” traumatic brain injury, in which repeated head hits can cause concussive injury yet are often not diagnosed and do not show any immediate clinical symptoms. We found, even in this very early stage of a mild head injury (prior to any disease), that football
players show disruption of the BBB, whereas control patients show intact, healthy BBB (Figure 6). In the realm of age-related dementia, we performed imaging in aging patients exhibiting mild cognitive impairment (MCI). Importantly, MCI represents an early stage of disease, in which subtle cognitive impairment can be detected via neurological mental status testing, yet patients typically do not exhibit any strong deficits that interfere with their daily lives; as such it may be the earliest stage at which future risk for dementia can be currently detected. Here again at this early stage, we show BBB disruption in affected patients that is absent in healthy individuals (Figure 7). These data show that at the earliest stages that disease risk can be detected, patients already show a disruption of the BBB, demonstrating the potential of this diagnostic to identify at-risk patients before pathology progresses to a critical stage.

In parallel with advancing our therapeutics, our plan is to continue to develop these diagnostic software resources as a user-friendly, diagnostic package that can be used with existing MRI infrastructure, and which we will release as freely available to clinicians (using an open source or freeware model). We believe that providing clinicians with the tools to diagnose BBB pathology will greatly improve understanding of when and how to prescribe our preventative therapeutics, providing a key step in improving patient outcomes by guiding treatment to the right patient at the right time. More broadly, we believe free availability of these diagnostics will greatly broaden the clinical understanding of the role of BBB pathology in causing disease, thus helping to contribute to a shift in clinical practices in which preventative approaches become the treatment norm.

Specific aims: to complete pre-clinical safety and proof-of-concept studies, positioning us to start clinical trials for preventative treatment in our target diseases.

Our studies to date have shown a very strong proof-of-principle: in rodents, treatment with our therapeutics are effective in preventing pathology arising from pathological, inflammatory TGFβ signaling, including epileptogenesis after BBB disruption and cognitive dysfunction and seizure vulnerability in aging mice. These studies were performed in the academic context, and reveal the fundamental role of BBB pathology in causing neural dysfunction and neurodegenerative disease. In creating our start-up, Envivo, our primary objective has been to bring our innovative approach to market, and our main current goal is to raise early stage funding that allows a newly innovating company to pass its first milestones. In our case, while we continue to maintain a close partnership with the fundamental research in the lab of Dr. Daniela Kaufer at UC Berkeley, external funding will allow us to complete the necessary studies that are not performed in an academic context: the nitty-gritty proof-of-concept studies that are a key step in demonstrating the feasibility, and mitigating the risk, of taking this technology from the lab and towards clinical trials. These include characterization and validation of the drugs for clinical use (dosage studies establishing minimum effective dose, duration of treatment efficacy, etc.) and safety studies (toxicology, side effects, etc.).

We will complete these studies using a virtual business model via contract research organizations (CROs) specializing in preclinical studies. The CRO approach carries many advantages, and mitigates a number of risks, as follows: 1) CRO best practices adhere to robust pre-clinical standards and carry the inherent advantage of site replication. As such, validation of our therapeutics from an external CRO will provide compelling evidence to future investors, granting agencies, regulators, and other stakeholders of the overall efficacy of our drugs, outside of our own lab. 2) As an early preclinical company, use of CROs will keep us lean and avoid any permanent infrastructure or personnel burdens at time when we are not expecting to generate revenue. 3) Contracting these preclinical studies will allow us to devote our sustained focus on the other key start-up milestones of our company: implementing strategy, securing funding, and developing partnerships towards clinical trials, thus avoiding a common start-up pitfall of getting lost in the weeds.

Pre-study assessment: The strength of our approach lies in a novel and highly disruptive preventative treatment directed towards broad markets of chronic, incurable diseases. This arises from our background as basic researchers, and we acknowledge that we are not experts in pre-clinical and clinical trials. However, we have recently assembled a strong team to improve our clinical expertise, including adding Drs. Michael Rogawski, Robert Knight, and Andrew Dillin as board members and scientific advisors. Furthermore, we have access to a large network of advisors via the QB3 institute
at UC Berkeley, which provides an array of programs and contacts to facilitate translation and spin out of university research into start-ups. Thus, as we move towards preclinical studies, we will first perform a comprehensive review of our research strategy with inside and outside advisors and consultants, and revise our approaches accordingly. As necessary we will also partner with or hire personal with relevant domain expertise. With those limitations in mind, the following specific aims represent the overall strategy of what we seek to accomplish in pre-clinical studies, and how they fit into our overall milestones.

**Specific aim 1: dose response and efficacy**

Our research has shown that a standardized dose of losartan (100 mg/kg) or IPW (20 mg/kg) is effective in preventing pathological TGFβ signaling leading to neural dysfunction, and we have also characterized IC50 and PK of IPW. Prior to proceeding with toxicology analysis and ultimately bringing these therapeutics to clinical trials, it will be necessary to establish the minimum effective dose of these drugs in preventing the target pathologies of epileptogenesis (PTE) and age-related cognitive decline, as well as frequency of dosing relative to the target disease. Establishing correct dose will allow us accurately assess subsequent toxicology and avoid potential pitfalls of failing toxicology due to excessive dose.

**SA1.1: Dosing for post-traumatic epilepsy.** One strong advantage of our preventative approach is that our therapeutics need only be given after acute injury, while BBB is disrupted, to prevent subsequent disease — for example 3 weeks of oral losartan prevented epilepsy after BBB disruption (Fig 3). This allows us to avoid one of the most common causes of toxic side effects, arising from long-term, chronic dosing. For losartan and IPW, we will use two mouse models to test efficacy at three dose concentrations. The disease models will be (1) direct infusion of albumin into the ventricles (ICV) via osmotic pump and (2) traumatic brain injury via the weight drop model, which has been shown to cause BBB breakdown. For each model, the following dosing timeframes will be implemented: 1, 2, and 3 weeks daily oral dose (gavage) concurrent with start of injury. Endpoint measures will be detection of seizures via electrophysiology (i.e. identifying minimum dose to prevent seizures), and at conclusion of the study histological analysis will be performed on the brains to quantify levels of inflammatory TGFβ signaling (levels of phosphorylated Smad2, the second messenger activated by TGFβR). Once minimum dose is established, a follow up study will be conducted in which dosing is initiated 2 days after injury, testing the clinical model in which a patient may first be acutely treated in ER, and only subsequently be diagnosed and treated with our therapeutics.

**SA1.2: Dosing for age-related dementia.** We have found that BBB disruption and pathological symptoms are present in aged mice reliably by 18 months of age. Thus we will test dosing in 18 month aged mice, with 3 dose concentrations, administered as an oral daily dose for 3 weeks. Outcomes will be cognitive performance in contextual fear conditioning and novel object recognition tasks, assessed at 0, 2, and 4 weeks after the end of dosing. Testing at the delayed timepoints will determine whether efficacy of therapeutics are lasting, or whether they require chronic dosing to ameliorate symptoms. Once minimum dose is established, a follow up study will be performed using a two-week-on, two-week-off dosing strategy (based on Eli Lilly molecule, LY2157299, in phase II for hepatocellular carcinoma), which has been an effective means of maximizing efficacy while minimizing toxicity in other therapeutic approaches.

**Specific aim 2: safety and toxicology**

We included losartan in our patent filing to enable a potential route for a rapid entry into market via new use of an existing drug that has already cleared FDA regulatory hurdles. If our dosage studies show an effective dose similar to that already used for clinically prescribed losartan, then we can rely on established safety. Thus, our toxicology studies will focus on IPW.

**2.1: Enabling studies – preliminary toxicology in rodent and non-rodent.** We will conduct two-week administration studies, at three dose concentrations, in rodent and non-rodent (dog) models, with cohorts of 3 males and 3 females, with specific focus on cardiovascular safety. Given that cardiovascular complications are the most frequent reason for candidate drug safety failure, this preliminary toxicology will allow assess the most likely setback before investing in full
toxicology. However, while cardiovascular toxicity is frequently observed in preclinical studies of TGFβ antagonists, it is not recapitulated in human clinical use with small molecules or antibodies 30.

2.2: Good Laboratory Practice (GLP) Toxicology. If our enabling studies are successful, we will proceed with full GLP safety studies. Studies will be 4 weeks of drug administration (compared to controls) with subsequent recovery in rodent and dog, under documented GLP practices. Doses will be low (no adverse effect), clinical, and maximum tolerated, as optimized by the prior studies. Target endpoints will be general toxicology, including mortality, clinical signs, body weight, temperature, activity level, hematology, clinical chemistry, toxicokinetics, and pathology (complete battery of tissues).

Business Plan

A major strength of our company is our connections to the academic lab of Dr. Daniela Kauf er and the supportive institutional environment at UC Berkeley, which facilitates entrepreneurship spun out of academic research. Thus, while using external funding for preclinical studies, we are positioned to simultaneously advance other aspects of our business plan in parallel with the support of University resources. This allows us to move forward at very low costs and with unique resources bolstering our success. Key resources are as follows: (1) The academic research lab: All of our preliminary data was generated in the lab of our advisors (Kauf er and Friedman), and these robust research programs will continue in parallel. In particular, future studies in progress include new clinical rodent disease models (TBI induced by weight-drop), rodent MRI to track BBB disruption and efficacy of treatment in real time, and continuing human studies to test the predictive power of BBB diagnostics. These studies will provide key data supporting our approach, and are performed from academic funding sources (grants, foundations, etc.). The lab also provides us a partner for our future STR program (to be submitted in next cycle). (2) Entrepreneurship programs: We are enrolled in the QSB Institute’s “Startup In-A-Box” program, the Bakar Entrepreneurial Fellowship program, and in the SkyDeck incubator space. These programs offer a vast array of resources to aid spin-out start-ups, including free incorporation and legal advice, free FTO analysis and market analysis, SBIR/STTR workshops, dedicated support staff, and access to development events including a large network of investors and consultants. We are also enrolled in UC Berkeley’s “Methods of Technology Innovation” (MTI) program, a crash course in entrepreneurial and business models for STEM grad students outside of the traditional MBA program, taught by Prof. Khag Sidhu, Chief Scientist & Founding Director of the Sutardja Center for Entrepreneurship & Technology. (3) IP: UC Berkeley’s IP office (IPRA) allows us a free path to file new patent disclosures derived from lab discoveries, which will then be licensed to use for our exclusive use. This allows us to obtain IP protection for future disclosures at no cost (in particular we are developing alternate TGFBR blockers, now in the synthesis and testing phase, as risk mitigation in case our current approaches fail preclinical hurdles.

Appendix  | References Cited

7. Shloufberg, D., Benito, M., Kauf er, D. & Friedman, A. Blood-brain barrier breakdown as a therapeutic target in
Appendix | Supplementary Data

Appendix 1. (A) Immunofluorescent staining in cell culture model shows that albumin (Alb-555) is taken up by cultured astrocytes (GFAP), leading to phosphorylation of the TGFβ messenger protein Smad-2. Treatment with therapeutic blockers (IPW, SJN, or losartan (Los)) prevents the inflammatory TGFβ signaling. (B) Quantification and verification with Western blot for pSmad-2 protein.
Appendix 2. (A) Albumin (red) is delivered into the brain ventricles by surgical implantation of intracerebroventricular (ICV) osmotic minipumps, and diffuses into the hippocampus where it is taken up by astrocytes (green). (B) Astrocytes (GFAP) take up albumin (BSA) within 48 hours. Activated astrocytes release inflammatory TGFβ cytokine (magenta, right image), and this inflammatory signaling is blocked by IPW or losartan.
Appendix 3. The common mechanism of inflammatory TGFβ signaling is induced across a variety of injury models involving disruption of BBB. Microarray analysis quantifies the changes in expression of genes in the TGFβ and subsequent inflammatory signaling pathways, as well as genes involved in reactive astrocytosis. Activation of this core pathway is seen in each injury model: undercut (UC; direct surgical damage to cortex); stroke (induced stroke in the hippocampus); DOC (induced breakdown of the BBB in cortex); Albumin (albumin treatment directly onto cortical surface); and TGFβ (treatment with the canonical ligand of the TGFβ pathway, TGFβ1, directly onto cortex). Upregulation for each gene (relative to control expression) is shown in red, downregulation is shown in blue.
Appendix 4. Our collected data shows the overall timeline of albumin induced pathology. In the first hours, albumin is taken up by astrocytes, which become reactive and activate the inflammatory TGFβ signaling pathway, releasing cytokines into the brain that alter neural function and connectivity. Neurons respond by increasing excitatory synaptogenesis, increasing expression of ion channels regulating excitability, while decreasing expression of genes involved in inhibitory neurotransmission. The net result is an increase in the excitatory/inhibitory balance in neural networks, leading to spontaneous epileptiform activity and epilepsy.
Appendix 5. Albumin, delivered by ICV pump, causes aberrant increases in neurogenesis in the hippocampus, quantified at 7, 14, and 30 days after albumin treatment (via labeling of the proliferation marker PCNA). The newly generated neurons show aberrant morphology, with lateralized dendrites indicating altered connectivity. This corresponds with other studies showing that aberrantly generated neurons in epilepsy models become hyper-connected and play a key role in generating seizures.
Appendix G. (A) Immunostaining shows that aged mice with BBB breakdown (see also Figure 5) have activation of inflammatory TGFβ signaling (green), which is absent in young adult mice. (B) Quantification of albumin in young and old mice shows that albumin is present throughout hippocampal brain regions of aged mice (ML – molecular layer, Hilus, CA1 and CA3), but is totally absent from young mice.
Appendix 7. Neural dysfunction and cognitive decline after pathological activation of the TGFβ signaling pathway is associated with increase in the slope of theta activity recorded from mice via in-vivo telemetric electrocorticography (ECoG). Control and young mice show normal brain activity, while mice treated with TGFβ1 or albumin, aged mice, or mice with induced inflammation (via the inflammatory molecule interleukin-6 (IL-6), all show altered theta slope. Critically, treatment with SJN, a blocker of the TGFβR, prevents altered neural activity.
Appendix B. Diagnostic MRI imaging is used to detect contrast agent that diffuses into the brain through disrupted BBB (whereas intact BBB prevents contrast agent from leaving the blood vessels). Here we use Ablavar, a contrast agent binds to albumin, and thus is carried into the brain with albumin as it crosses the disrupted BBB.
Appendix 9. Our therapeutic model. By administering IPW, we block the initial pathological signaling events leading to neural reorganization, neurodegeneration, cognitive decline, and epilepsy after BBB disruption.