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Cumulative and Persistent Changes in the Brain in Response to Stress and Aging

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Cumulative and Persistent Changes in the Brain in Response to Stress and Aging

by

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Abstract

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The brain has a remarkable capacity for plasticity: it physically encodes lived experiences within itself, and so is constantly sculpted and shaped by the environment. On short time scales, specific experiences and events are embedded as structural changes at the level of the synapse – in other words, memory. Over longer time scales, other forms of plasticity are involved in causing cumulative and persistent structural changes that do not necessarily represent specific information or experiences per say, but rather influence the overall behavioral mode of the organism. Such changes are not limited to the synapse, but rather may involve broad changes in the cellular composition and structure of the brain. This work seeks to characterize such mechanisms of long-lasting plasticity, focusing on two major areas. Firstly, we investigate how chronic stress affects the cell fate of neural stem cells in the adult hippocampus to cause an increase in the production oligodendrocytes and a decrease in neurons. By changing the cellular composition of the hippocampus, this shift towards oligodendrogenesis may provide a structural basis for long-lasting changes in mood and behavior that can occur after stressful experiences. Secondly, we investigate biological changes in the aging brain, seeking to uncover mechanisms that are involved in cognitive decline. We find that aging involves a loss of integrity of the vascular blood-brain barrier, which allows molecules from the blood to enter into the brain. In turn, this triggers an inflammatory response the causes neural dysfunction and induces cognitive decline. Together, these studies seek to broaden the time scales on which we observe and characterize brain plasticity. By understanding persistent changes that accumulate in the brain over months or years, we may provide new insights into complex neurological diseases that manifest slowly in response to lifestyle risk factors, and ultimately develop new, more effective treatments.

Table of Contents

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Chapter 1. Introduction

Adapted from: Friedman AR and Kaufer DK (2014). "Emerging Roles for Hippocampal Adult Neural Stem Cells in Memory." Colloquium Series on Stem Cell Biology, Lecture #5. Morgan and Claypool Life Sciences Publishers, San Rafael; from: Chetty S*, Friedman AR*, Taravosh-Lahn K, Kirby ED, Mirescu C, Guo F, Krupik D, Nicholas A, Geraghty AC, Krishnamurthy A, Tsai MK, Covarrubias D, Wong AT, Francis DD, Sapolsky RM, Palmer TD, Pleasure D, and Kaufer D, 2014. Stress and glucocorticoids promote oligodendrogenesis in the adult hippocampus. *Mol Psy*, 19(12):1275-83; and from: Senatorov VV Jr*, Friedman AR*, Milikovsky DZ, Ofer J, Saar-Ashkenazy R, Charbash A, Jahan N, Chin G, Mihaly E, Lin JM, Ramsay HJ, Moghbel A, Preininger MK, Eddings CR, Harrison HV, Veksler R, Becker A, Hart B, Rogawski MA, Dillin A, Friedman A, and Kaufer D, 2018. Blood-brain barrier breakdown during aging causes chronic but reversible neural dysfunction via TGF-beta signaling. Manuscript in preparation. *Equal contribution.

The greatest unknowns in biology remain the things that we cannot see, and perhaps the greatest of these is time. Consider that nearly every experiment in the history of biology has been based on observing a "snapshot" of a single moment in time, and each generation's greatest breakthroughs have been akin to increasing the resolution of those snapshots, yet have fallen short of achieving something akin to video that could observe how changes occur over time. For example, genome sequencing allowed us to see all of the complexity that arises from the genes of an organism. Yet it did not allow us to see how cumulative and subtle experiences like physical injuries, environmental exposures, and social experiences lead to individual differences in gene expression over time that profoundly dictate life trajectories and health outcomes. In current neuroscience, new technologies reveal the thousands of connections that a neuron makes, and the firing patterns of functional neural networks that underlie our most personal thoughts, memories, and emotional experiences. Yet they do not allow us to see how previous experiences are embedded to cause long-lasting changes in behavior. How, for example, early life trauma leads to life-long changes in cognitive, emotional or social behavior that not only profoundly affect personal experience but also define resilience vs. vulnerability to mental illnesses; and how single or cumulative injuries in the brain can trigger slow and progressive changes that only years later manifest as diseases such as epilepsy or dementia.

And so it is because we have so few tools to "see" biological changes over time that one of the greatest questions of biology – how do individual outcomes arise from different experiences over the lifetime of an organism? – remains almost completely unanswered. And some of the greatest biological problems of our time – such as those of biologically-driven cycles of life trauma and mental illness; and of progressive neurological diseases triggered by life experiences – remain completely unsolved. In light of these challenges, my graduate research has been devoted to studying how life experiences are embedded in the brain to cause long-lasting changes in health and disease. The common thread in my collected body of work is discovering new, unexpected mechanistic perspectives on human neurological health problems that develop slowly over long time periods, and then on using those insights to guide new, interventional approaches for improving outcomes.

Because we lack technological tools akin biological "video," I have instead tackled these questions by assembling a series of "snapshots" of the brain, a sort of biological "flipbook." In particular, my work has focused on how the hippocampus changes over critical periods of time that are associated with the genesis of disease, including after stress and during aging. I focus on the hippocampus because it is one of the most plastic and malleable parts of the brain – the region where the memories of our lived daily experiences are physically encoded; where new neurons, born from neural stem cells, generate new neural connections in the adult brain (at least in rodents[1,2]); and where the potency of the stress response, and of other types of emotional salience, are regulated to help us adopt behavioral modes that are matched our surroundings, helping to create the range of lived experiences from utmost vigilance to absolute chill. Likely related to its unusual plasticity and centrality in all brain networks, the hippocampus is also the focal point for a wide range of progressive neurological disease: it is a trigger point for traumatic memories in post-traumatic stress disorder and depression[24]; it is the circuit that most commonly runs amok in epilepsy, sending paroxysmal, excitatory waves of neural firing that propagate through the brain; and it is one of the regions most affected by Alzheimer's disease and other forms of neurodegenerative cognitive decline – a slow erosion of the neurons that allow us to form and retrieve memories. In this sense, the hippocampus is inseparable from our individuality. In health, it is part of the essential neural substrate that makes us who we are, and when afflicted by disease, it may cause us to lose not only cognitive function but essential aspects of our experiential and emotional selves. In all of these cases, both health and disease, the hippocampus works on slow time scales, be it the gradual, lifelong accumulation of memories, the cumulation of psychological trauma, or the decline of aging. To truly understand these phenomena, we too must slow our perspective, stepping outside the bounds of single-timepoint experiments, and stretching into a long view investigation of how the brain changes over the time – ideally over the entire lifespan.

1.1 Hippocampal neural stem cells and long-lasting plasticity after stress

Within the hippocampal dentate gyrus, a unique population of neural stem cells (NSCs), located at the SGZ border between the granule cell layer and the hilus, gives rise to thousands of new cells daily[3,4]. The development of these NSCs, from multipotent stem cell to fully mature differentiated cell, can be conceptualized as progressing through several stages. The "true" stem cells of the SGZ are generally in a quiescent state, such that only a small fraction of the population undergoes cell division while the majority remains inactive. In this quiescent state, the NSC has a radial glia-like morphology, with a single process extending through the granule cell layer and terminating with dendritic-like branching in the molecular layer, among the dendrites of mature granule cells. At any given time, a small percentage of the NSC population leaves quiescence and begins to proliferate, undergoing mitotic division to produce two daughter cells. These daughter cells can adopt several different cell fates, demonstrating the multipotency and self-renewal capacity of the NSC population: one or both daughter cells may retain a radial glia-like fate, allowing for symmetric or asymmetric maintenance of the NSC population; or daughter cells may become intermediate precursor cells (IPCs), which undergo additional divisions before differentiating to produce a large number of new cells; or daughter cells may directly differentiate into mature cell types. Thus, a single NSC division event produces a small cluster of

new cells, ranging from two to dozens (due to transient amplification of IPCs), most of which will ultimately turn into new neurons or astrocytes[5,6].

After proliferation, the new cells generated by RGLs and IPCs undergo a process of maturation and terminal differentiation into one of several brain cell types: granule neurons, astrocytes, and possibly oligodendrocytes (see chapter 2). The majority of IPCs differentiate into neurons; because this IPC population is highly proliferative, a large number of new neurons are produced daily. In contrast, astrocytes appear to be primarily generated from RGLs at low numbers. Thus, under baseline physiological conditions, the relative output of differentiated new cells is approximately 70-90% neurons and 10% astrocytes[6,7]. However, two caveats complicate the quantification of cell fate. Firstly, new neurons undergo a process of selective pruning, in which only a small subset of cells are incorporated into the network, while the rest undergo programmed cell death. Thus, many more new neurons are produced than will ultimately survive and incorporate into functional networks. The second caveat concerns oligodendrocytes: while NSCs cultured in vitro produce oligodendrocytes, this has not been clearly documented in endogenous NSCs in vivo. Furthermore, a distinct population of oligodendrocyte precursor cells (OPCs) also generate new oligodendrocytes in the dentate gyrus, and their output cannot be distinguished from NPC oligodendrogenesis without careful lineage tracing.

The functional impacts of new neurons are most well-understood on the fast time scale, in which they can become rapidly recruited by a single stimulus that a rodent experiences, and through mechanisms such as LTP appear to contribute to the formation of novel "memory traces" that encode the representation of that particular experience[8–12]. In other words, new hippocampal neurons are thought to help quickly encode experiences into durable memory representations. This occurs in part because new neurons pass through a critical period of enhanced excitability, coinciding with their formation of functional connections within the existing neural network[10,13–15]. This critical period appears to allow the new neurons to become "tuned" to the particular stimuli that the animal was experiencing during that time, allowing the new neurons to serve as a "blank slate" to help encode the unique features representing a particular place in time.

New neurons also appear to have roles in regulating emotional responses that may operate on slower, more long-lasting time scales. For example, the overall level of neurogenesis is highly regulated by the environment, with stress decreasing neurogenesis while enriched environment increases neurogenesis[16]. These changes in cell proliferation occur before the cells are functionally relevant, meaning that there is a time delay from the point of proliferative changes to the point where those cells mature and begin to participate in the network. This raises a pressing question that to date has been almost completely unanswered: what are the actual adaptive and functional effects that arise from changing the level of neurogenesis? One possibility is that overall changes in cell number may not affect response to a given stimulus, but rather change the general behavioral state of the animal, perhaps by changing the number of neurons that are available in (and hence affect the cognitive capacity of) functional sub-regions of the hippocampus. Indeed, experimentally altering the overall levels of neurogenesis has been shown to change rodent emotional responses, including stress response, anxiety, and depressive-like behaviors[9,17–20].

Such structural changes in the composition of the hippocampus may be particularly relevant to stress, which can induce long-lasting changes in mood and behavior. Stress is a risk factor for a variety of mood and anxiety disorders, including depression and post-traumatic stress disorder (PTSD), which can manifest years after the stressful event[21]. The mechanisms that account for this persistent vulnerability are not fully understood. While many effects of stress on the brain are relatively transient – involving the actions of glucocorticoid (GC) stress hormones on mineralcorticoid and glucocorticoid receptors (MR and GR) to regulate short-term physiological responses[22,23] – long-term effects have also been identified. For example, stress affects long-term potentiation (LTP), and epigenetically regulates the hypothalamic-pituitary-adrenal axis[24,25]. However, mechanisms by which stress may lead to long-lasting structural changes in the brain have not been thoroughly explored. There are hints that GCs may directly affect white matter structure by regulating oligodendrogenesis. In oligodendrocyte precursor cell (OPC) culture, GCs are potent inducers of pro-oligodendrogenic transcription factors and increase oligodendrogenesis[26–28] and myelination[29–34]. GCs also dysregulate myelination in utero[35]. In the periphery, myelinating Schwann cells similarly show responsiveness to GCs, including increased myelination in vitro and in vivo[36,37]. Interestingly, changes in white matter have been documented in many brain regions in a variety of stress-related mental illnesses, including PTSD, schizophrenia, autism and depression[38–43]. Together, these data suggest the unexplored possibility that stress may cause dysregulation of oligodendrogenesis, which may create a persistent, white matter structural vulnerability to mental illness.

1.2 Cognitive decline in the hippocampus during aging

The advance into old age involves progressive brain dysfunction[44,45], with considerable variability in individual trajectories. While some individuals remain healthy and unimpaired throughout the lifespan, most aging humans proceed into mild cognitive impairment, and smaller subsets develop severe neurological diseases including epilepsy, dementia, and neurodegenerative disorders. Comparative investigation in rodents, monkeys, and humans, has revealed a set of common hallmarks of early aging that are remarkably similar across mammalian species, including downregulation in neurotransmission proteins, dysregulation of neural signaling, and loss of synapses in key cognitive structures, particularly in the hippocampus and cerebral cortex[44,45]. These findings suggest that there may be conserved regulatory pathways that control neural activity and plasticity to enact the stereotypical “first steps” in the progression from a healthy to aged brain. Furthermore, such fundamental changes in neural function could themselves plausibly contribute to a range of symptomatic outcomes associated with age-related disease. For instance, imbalance in the strength and connectivity of the excitatory and inhibitory components of circuits could lead to dysregulated neural firing causing errors in signal processing and cognitive impairment, but also to loss of inhibitory control and hyperexcitability contributing to risk for seizures and epileptogenesis. Yet while there has been extensive characterization of canonical, disease-specific mechanisms associated with age-related dementia – such as the biochemical steps involved in generating the aggregating form of the protein Aβ – there is as yet little understanding of the regulatory pathways that underlie dysfunction in neural circuits during natural aging.

The blood-brain barrier (BBB) forms one of the most essential and tightly-regulated interfaces of the body. Composed of specialized endothelial cells, pericytes and astrocytic end-feet that sheathe the brain capillaries, the BBB restricts the diffusion of molecules from the blood to create a highly privileged and controlled environment that is absent in other tissues. As such, the BBB establishes the exacting milieu that enables brain function, including the precise ionic concentrations needed for neural activity, the compartmentalization of brain-specific growth factors and signaling molecules, and the immune-privileged brain environment[46,47].

As early as the 1970s, clinical researchers began to report alarming observations of BBB dysfunction in aging patients[48], raising the possibility of a new form of biological senescence involving vascular leakiness, which would be expected to cause neurological complications[47,49–52]. While these reports generated controversy, including debate over how to accurately measure BBB status in living subjects[53,54], meta-analysis across clinical BBB studies concluded that BBB dysfunction is not only widely prevalent in aging individuals, but also strongly correlated with neurological disease[55], including Alzheimer's disease[52,56–58]. Furthermore, BBB dysfunction in aging patients was found to be highly associated with mild cognitive impairment when localized in the hippocampus[59]. This regional specificity suggests that vascular leakiness may directly cause impairments in affected brain regions. These clinical findings raise the possibility that BBB dysfunction may be a major contributor to age-related neurological diseases including dementias[59]. Yet remarkably there have been very few animal studies investigating BBB dysfunction during aging.

To identify candidate mechanisms through which BBB dysfunction might cause age-related impairments, we turned to other disease contexts where the role of BBB disruption has been studied more extensively. Head trauma causes pronounced BBB dysfunction, which can persist for months or even years after the initial injury[60–62]. About 5-40% of traumatic head injury patients go on to develop secondary post-traumatic epilepsy (PTE) after a silent period of epileptogenesis[63]. In rodent studies, we previously found that inducing BBB disruption in otherwise healthy animals is sufficient to cause epileptogenesis via the signaling action of blood molecules that leak into the brain, in particular blood serum albumin[63]. Following BBB disruption, albumin induces an inflammatory TGFβ signaling cascade, primarily by TGFβR binding and endocytosis into astrocytes[64–66]. In turn, activated astrocytes produce pro-inflammatory cytokines, including TGFβ1 ligand (thus amplifying the TGFβ signaling cascade), IL-6, and IL-1β[64,67,68], propagating a signaling response that enacts physiological changes in neighboring neurons – including changes in expression of genes governing neurotransmission and excitability/inhibitory (E/I) balance, and alterations in connectivity and plasticity[64,67,69,66,70,71]. This reorganization of local neural networks, and associated neural dysfunction causes lower seizure threshold and spontaneous seizures[64,67,66], as well as delayed reduction in dendritic arborization and cell loss[72].

These studies provoked us to investigate whether BBB dysfunction, and consequent activation of TGFβ signaling, may play a similarly central role in the neurobiology of aging. In further support of this candidate pathway, TGFβ signaling is elevated in aging rodents[73–77] and human AD patients[78–80]. However, the cause of this elevated signaling, and whether it is beneficial or pathological, has remained controversial and poorly understood. For example, elevated TGFβ signaling from activated astrocytes increases pathological outcomes in various

disease models, including Alzheimer's disease[81–83], ALS[84,85], and age-related dysfunction in hypothalamic glucose homeostasis[86]. In contrast, other studies have suggested that TGFβ signaling promotes neural survival in the aging brain[87–89]. These conflicting results may arise from the fact that the TGFβ signaling pathway is a ubiquitous and pleiotropic regulatory pathway, which exerts a wide variety of effects that are specific to cell-type and context. Here, we resolve this controversy by exploring the hypothesis that BBB dysfunction is itself a trigger of TGFβ signaling in aging, and that in turn this TGFβ signaling transduces an inflammatory cascade that directly causes pathological outcomes associated with neurological decline in aging.

Chapter 2. Stress and glucocorticoids promote oligodendrogenesis in the adult hippocampus

Adapted from: Chetty S*, Friedman AR*, Taravosh-Lahn K, Kirby ED, Mirescu C, Guo F, Krupik D, Nicholas A, Geraghty AC, Krishnamurthy A, Tsai MK, Covarrubias D, Wong AT, Francis DD, Sapolsky RM, Palmer TD, Pleasure D, and Kaufer D, 2014. Stress and glucocorticoids promote oligodendrogenesis in the adult hippocampus. *Mol Psy*, 19(12):1275-83 *Equal contribution.

2.1 Introduction

The hippocampus is a key structure regulating memory and emotion, and plays a role in a variety of emotional disorders[90]. Changes in hippocampal cell composition therefore could have dramatic implications for vulnerability to and progression of mental illness. In particular, the DG contains a unique population of neural stem cells (NSCs) that proliferate throughout adulthood, and have functions in regulating negative affective states including stress response, fear, anxiety, and depression[9,19,91,92]. NSCs are multipotent, producing neurons and glia, but their potential to generate oligodendrocytes in vivo is debated[5]. Resolving this ambiguity may reveal new insight into NSC function, since alteration of the oligodendrogenic component of NSC output could provide a novel pathway through which stress affects white matter composition and hippocampal function. In this study, we sought to determine whether stress and GCs affect oligodendrogenesis in the adult hippocampus, and specifically if the oligodendrogenic potential of NSCs is modulated by GC exposure.

2.2 Results

Stress and GCs increase oligodendrogenesis in the hippocampus

Studies investigating the effects of stress on NSCs in the adult hippocampus have primarily focused on the extent to which new neurons are generated. However, new glial cells are also generated in the hippocampus and may similarly be susceptible to environmental factors and hormones. Here we investigated whether stress and stress hormones (GCs) alter the oligodendrogenic potential in the adult hippocampus. To assess these effects, we subjected rats to 1 week of immobilization restraint stress and subsequently quantified the percentage of newborn cells (marked by BrdU, Figure 2.1A) in the hippocampal DG that adopted a neuronal or oligodendrocytic cell fate. In agreement with previous studies[93,94], stress significantly decreased the percentage of BrdU+ cells that co-labeled with the early neuronal marker Tuj1 (Figure 2.1B and C). Interestingly, stress significantly increased the percentage of BrdU+ cells that co-labeled with the mature oligodendrocyte marker MBP (Figure 2.1B and C), revealing that stress increases oligodendrogenesis in the DG. To determine if an increase in circulating GCs is sufficient to upregulate hippocampal oligodendrogenesis, we next subjected rats to daily injections of cort (40 mg/kg, comparable to elevated serum GC levels induced by immobilization stress[95–97]) or vehicle for 1 week (Figure 2.1D) and assessed the neurogenic and oligodendrogenic potential within the DG. Similar to stressed rats, cort-injected rats showed a decrease in the percentage of new neurons, and an increase in the percentage of new oligodendrocytes, relative to controls (Figure 2.1E). When rats were allowed to recover from

cort injections for 1 week, the percentage of new neurons normalized to control levels did not differ between cort and vehicle injected rats (Figure 2.1F), consistent with prior studies on the long-term changes in neurogenesis following recovery from GC exposure[98,99]. In contrast, the percentage of newborn oligodendrocytes in cort vs. vehicle injected rats remained significantly higher and persisted even following recovery from cort injections (Figure 2.1F).

GCs increase oligodendrogenesis from NSCs in vivo

While NSCs produce neurons, astrocytes, and oligodendrocytes in vitro[100], a recent study reported that they do not produce oligodendrocytes in vivo[5]. If true, this would suggest that hippocampal oligodendrogenesis arises exclusively from a distinct population of OPCs. An alternate possibility is that NSCs generate new oligodendrocytes at a level that is low under "baseline" conditions but higher in response to stress. To investigate this, we used a transgenic mouse (nestin-Cre ER^{T2} / RosaYFP)[101] expressing an inducible (fused to estrogen receptor variant ER^{T2}) cre recombinase enzyme driven by the promoter for the NSC marker nestin. In this system, induction with the ER^{T2} agonist tamoxifen causes recombination leading to constitutive expression of the YFP marker in nestin-expressing NSCs, allowing for unambiguous analysis of their cell fate. After tamoxifen induction to initiate nestin-driven NSC labeling, mice were injected with cort (25 mg/kg) or vehicle for 10 days and assessed for oligodendrogenic potential 7 days later (Figure 2.2A). Quantification of the percent of NSC derived cells (YFP tagged) that co-labeled with the oligodendrocyte marker GST-π showed that NSCs produced a low number of new oligodendrocytes under control conditions, and that stress significantly increased oligodendrogenesis from NSCs (Figure 2.2B-F).

GCs increase oligodendrogenesis from NSCs in vitro

Adult hippocampal NSCs can be cultured in a proliferative state in vitro, and can be induced to terminally differentiate by withdrawal of fibroblast growth factor 2 (FGF-2) from the culture media[100], thus providing a useful system to investigate the direct effects of cort on NSC fate. We treated NSCs with 1 μM cort or vehicle for 75 hrs (under low FGF conditions that permit unbiased differentiation) and determined the total number of new neurons (Tuj1+) and oligodendrocytes (MBP+) (Figure 2.3A). Compared to vehicle controls, cort-treated NSCs produced significantly fewer neurons (Figure 2.3B-C). Cort also caused a significant increase in the production of oligodendrocytes from NSCs, suggesting that cort can directly induce NSC oligodendrogenesis (Figure 2.3B-C). There was no significant difference in total cell numbers between cort and vehicle treated cultures (vehicle, 334.0±66.9; cort, 432.7±88.7; $p = 0.4$). To determine if cort-induced oligodendrogenesis is GR mediated, we blocked GR signaling by transfecting NSC cultures with the dominant negative GR (dnGR) viral vector[102]. Compared to NSCs infected with a control GFP vector, NSCs infected with dnGR and treated with cort showed significantly more neurons and fewer oligodendrocytes (Figure 2.3D and E), similar to baseline levels seen in vehicle-treated cultures. Thus, blocking GR function prevented the effect of cort on NSC oligodendrogenesis.

GCs promote an oligodendrogenic transcriptional program in NSCs

Cell fate programming is known to be regulated by complex interactions of multiple transcription factors. Oligodendrocytic fate is regulated by the pro-oligodendrogenic transcription factors Olig1 and Olig2[103–109], which are inhibited by Inhibitor of Differentiation 2 and 4 (Id2, Id4)[110]. To determine if GCs promote a pro-oligodendrogenic transcriptional program we performed RT-qPCR on NSC cultures treated with cort or vehicle for 48 hrs. There was no change in the expression of any neurogenic factors analyzed following cort treatment (Mash1 and 2; Neurogenin1, Ngn1; Sox21; and NeuroD1; Figure 2.4A). In contrast, Olig1 and Olig2 mRNA levels were dramatically increased and Id2 mRNA was significantly decreased following cort treatment, relative to control. The levels of Sox10 and Id4 mRNA did not change following cort exposure.

Blocking GR signaling by transfection with dnGR prior to cort treatment eliminated the GC-induced increases in Olig1 and 2 transcripts, and suppression of Id2, relative to GFP transfected controls (Figure 2.4B). These results demonstrate that the Id-Olig network is modulated by cort via a GR-dependent mechanism, suggesting that this transcriptional program is involved in GC-induced oligodendrogenesis.

Olig1 is a key regulator of oligodendrogenesis that acts as a nuclear-localized transcription factor when specifying oligodendrocyte differentiation and progressively localizes in the cytoplasm in mature oligodendrocytes[104,111,112]. Olig1 Nuclear localization is inhibited by the binding of Id2 and Id4[110], therefore the decrease in Id2 would predict an increase in functionally active nuclear Olig1. We assessed the level of Olig1 protein in total protein and in nuclear fractions of NSCs treated with cort, and found an increase in Olig1 protein in both total (Figure 2.4C-D) and nuclear (Figure 2.4E) fractions following cort treatment. We further assessed nuclear co-localization of Olig1 with BrdU *in vivo* following 1 week of GC injections or immobilization stress treatment. Both GC injections (Figure 2.4F) and stress (Figure 2.4G) resulted in an increase in nuclear co-localization of Olig1 and BrdU, relative to respective controls, in the DG. These results indicate that stress and GCs cause an increase in Olig1 protein levels in the nucleus both *in vitro* and *in vivo*, implicating Olig1 in GC-induced oligodendrogenesis.

2.3 Discussion

We demonstrate that stress and GCs increase hippocampal oligodendrogenesis *in vivo* and show that new oligodendrocytes can arise from nestin positive NSCs in the DG. Furthermore, we demonstrate a direct, GR-dependent effect of GCs on NSC differentiation into oligodendrocytes, which involves an upregulation of pro-oligodendrogenic genes, and increased nuclear localization of Olig1 protein. Current models for stress-induced emotional disorders suggest that previous stress experience can create a persistent vulnerability to mental illness that lasts many years beyond the stressful experience. Such models require mechanisms by which stress can cause long term changes in brain structure and function. Here we describe a novel mechanism of stress-induced oligodendrogenesis which may contribute to persistent changes in brain structure. In turn, increases in oligodendrogenesis could affect cognition in at least two ways: by altering the ratio of oligodendrocytes to neurons, and by altering myelination (white matter tracts).

Alteration of the oligodendrocyte:neuron ratio could affect cognition due to oligodendrocytic roles in synapse formation. Oligodendrocytes inhibit axon growth cones[113–117], and OPCs are both repulsive and nonpermissive for growing axons[118,119]. While the effects of changes in oligodendrocyte abundance on synaptic formation and plasticity are unknown, it seems likely that increased abundance would result in suppression of synaptogenesis. This suppressive effect, combined with the well-documented effects of GCs in reducing neurogenesis and new neuron survival[93,94,120], would be predicted to dramatically impair hippocampal function. In terms of myelination, increased stress reactivity in aging[22] correlates with increase in white matter in both humans[121–123], and monkeys[124]. Age-related cognitive decline also correlates with white matter increases in the brain[124,125]. Beyond cognition, a variety of mental health conditions[38], including depression, schizophrenia, PTSD, and suicide[39–42], are linked to changes in white matter abundance[43]. Interestingly, Cushing's syndrome and other diseases involving elevated cortisol levels are associated with alterations in affective and hippocampal function[126–128], though possible white matter contributions to these symptoms have not been investigated.

As the first report that stress hormones increase oligodendrogenesis in the adult hippocampus, this study opens avenues for additional investigation. Many species differences have been observed in hippocampal neurogenesis; for example rats produce more new neurons than mice, and those neurons mature more rapidly[3], while humans may produce fewer new neurons, if any[129,1]. We found stress induced-oligodendrogenesis in both rats and mice, though the different labeling techniques used in these species preclude direct comparison of oligodendrogenic rate. Key questions for future study involve whether effects of stress induced oligodendrogenesis can be generalized to other brain regions and other mammals (especially primates[130]), and if so whether there are important species differences; if increased oligodendrogenesis causes increased myelination; whether these effects persist and accumulate with stress load, and in particular whether stress-induced epigenetic changes[24,25,131] could target oligodendrocytic genes to persistently dysregulate white matter; and ultimately how changes in oligodendrogenesis affect neural function and behavior. Given that the effects reported in this study occur within the hippocampus, our results suggest a possible link between GC-induced oligodendrogenesis and memory function. Long term alterations in oligodendrogenesis and myelination could regulate memory retention and have important implications for disorders like PTSD. Stress has been shown to induce long-lasting changes on cholinergic neurotransmission and neuronal hypersensitivity as a result of genetic and epigenetic changes[132–134]. Whether stress-induced oligodendrogenesis plays a role in regulating neurotransmission signaling systems remains to be investigated and could potentially offer new targets for therapeutic interference. Furthermore, our findings raise the possibility that stress is an underlying factor in unexplained changes in white matter (leukoaraiosis) that are frequently observed in older patients and, in some studies, correlated with cognitive deficits[135,136].

There is also considerable current interest in understanding the functional role of adult hippocampal neurogenesis from NSCs. We show that cort increased oligodendrogenesis in NSC cultures in addition to decreasing neurogenesis, and used lineage tracing of nestin-expressing NSCs to show that cort also increases NSC oligodendrogenesis in vivo. These findings challenge the current thinking that hippocampal NSCs do not generate oligodendrocytes in vivo, and suggest that while NSCs generate few oligodendrocytes under basal conditions, exposure to

stress and GCs redirects the cell fate of differentiating NSCs towards oligodendrogenesis via a GR-mediated mechanism. The differences observed in the increases in oligodendrogenesis between exogenous and endogenous GCs (cort vs. stress treatment) may be due to differential GR/MR activation and/or variations in the rate of maturation of oligodendrocytes. Since Olig1 translocates to the cytoplasm as oligodendrocytes mature, the increased nuclear localization of Olig1 in BrdU+ cells in stressed animals relative to cort-treated animals may indicate variations in the rate of maturation of new oligodendrocytes. Overall, the data shows that GCs mimic the effects of stress and supports the conclusion that both immobilization stress or cort hormone injection produce an increase in oligodendrogenesis. The possibility that a GR-induced oligodendrogenic transcriptional program could override neural fate is supported by studies that have found that Olig1 promotes oligodendrogenesis in embryonic NSCs[137]. Our results suggest that, at the step of NSC differentiation and fate choice, neurogenesis may be inversely related to oligodendrogenesis. This finding advocates a shift in perspective on adult neurogenesis, which has largely focused on the proliferation, survival, and function of new neurons as a basis for cognitive changes associated with altered neurogenesis. We propose that changes in oligodendrogenesis, or in the ratio of glia and neurons, may contribute to alterations in hippocampal-dependent behavior. Possible roles for oligodendrocytes should be considered in previous and future studies that implicate neurogenesis in behavioral outputs. In particular, changes in neurogenesis have recently been implicated in emotional dysfunction, altered stress response, and depression[9,17–19,138]. However, potential contributions of concurrent altered gliogenesis to these emotional perturbations have not been considered. Overall, our results suggest a new model in which stress may contribute to persistent vulnerability to mental illness by inducing oligodendrogenesis. We speculate that accumulated stress load across the lifespan may alter the rate or extent of myelination to create a persistent, structural vulnerability to mental illness.

2.4 Methods

Cell culture conditions. Preparation of NSC culture was as previously described[139,140]. Cells were cultured (37°C, 5% CO2) on poly-ornithine (Sigma, St. Louis MO) and laminin (Invitrogen, Carlsbad CA) coated plates in N2-supplemented (Invitrogen) Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) (Invitrogen) with 20ng/mL recombinant human FGF-2 (PeproTech, Rocky Hill NJ). For differentiation studies, cells were cultured in low FGF-2 (0-5ng/mL) for 12-18h (200,000-300,000 cells per 6cm dish for RT-qPCR and 12,000-15,000 cells per coverslip in 24 well plates for immunocytochemistry (ICC), and treated with vehicle (EtOH, 0.1%), 1 µM corticosterone (cort), or 1 µM dexamethasone (DEX). Cells were treated every 24h for 75h with no media replacement for ICC, once for 48h for RT-qPCR, and once for 72h for Western blot. For dominant negative GR (dnGR) experiments, dnGR or GFP vector was added to cultures after cells were in differentiating conditions for 6-10h, with cort treatment 12h later. For GR ICC, NSCs were incubated with BrdU (30µM) 12h before fixation.

Amplicon construction of dnGR. dnGR was generated as previously described[102]. Briefly, a truncated rat GR (Genbank M14053; amino acids 1-745) was PCR amplified and ligated to the first 24 base pairs of the human GR-β isoform then transfected into E5 cells. Superinfection occurred 24 hours later with the helper virus d120 at a multiplicity of infection (MOI) of 0.3.

Amplicons were harvested by freeze-thaw lysis followed by PEG-IT (System Biosciences, Mountain View, CA) precipitation as per manufacturer's instructions. Pelleted viruses were resuspended in dH2O. Vector titers were 2-3x10^6 infectious particles/mL and d120 helper virus titers of 0.4 - 1.0x10^7 plaque forming units (PFU)/mL.

Real-time PCR. Real-time PCR was performed with an iCycler/iQ detection system (Bio-Rad, Hercules, CA). Primer pairs were used with SYBR green. Data were analyzed with the Relative Expression Software Tool. The ribosomal subunit, 18S, was used as an internal control.

Immunocytochemistry and in vitro quantification. NSCs were fixed in 4% paraformaldehyde (PFA) and immunostained with rabbit anti-GR (1:500, Thermo Scientific, Rockford IL), mouse anti-Nestin (1:500, BD Biosciences, San Jose CA), and rat anti-BrdU (1:500) overnight at 4°C, and then treated with Cy3 anti-mouse, FITC anti-rat, and Cy5 anti-rabbit (1:500, Jackson ImmunoResearch, West Grove PA) for 2h.

For differentiation studies, cells were immunostained (overnight, 4°C) with mouse anti-β-III tubulin (Tuj1; 1:1000, Covance, Princeton NJ) or rat anti-MBP (1:100, Abcam, Cambridge MA) followed by 2h incubation with Cy3 or FITC-conjugated secondary antibodies (1:500, Jackson). The nuclear stain DAPI was added prior to mounting coverslips in 1,4-diazabicyclo[2.2.2]octane (DABCO). Images were obtained with an inverted fluorescence microscope (20x objective; Zeiss, Oberkochen, Germany), and blind cell counts were taken using Metamorph software. 20 random visual fields were analyzed per coverslip. Data are the percentage of positive cells in relation to the total number of DAPI-stained nuclei present in the culture. All experiments were independently replicated at least 3 times.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Whole-cell proteins were extracted using Radio-Immunoprecipitation Assay (RIPA) buffer. Nuclear and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoSci). Protein concentrations for each sample were determined using bicinchoninic acid (BCA) Protein Assay Kit (ThermoSci). Proteins were separated in 8% or 10% polyacrylamide denaturing gels (for GR and olig1, respectively) and transferred to Immuno-Blot polyvinyl difluoride (PVDF) membrane (Bio-Rad), then incubated (overnight, 4°C) with anti-GR monoclonal antibody (PA1-510A; ThermoSci) at 1:500 dilution, followed by anti-mouse or anti-rabbit conjugated to horseradish peroxidase, or with primary anti-olig1 (rabbit anti Olig-1, 1:25000; from Charles Stiles, Harvard University), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000, Jackson; 1h, RT). Membranes were detected using a chemiluminescence kit (Western lightning Plus ECL, Perkin Elmer, Waltham MA) and quantified by densitometry. Membranes were stripped and reprobed using ß-actin antibody (1:3000; Sigma) to control for equal loading.

Animal treatments. Experiments used adult male Sprague-Dawley rats (2-3 mo; Charles River) pair housed, or C57Bl/6J transgenic nestin-Cre ER^{T2} / RosaYFP mice[101] (2-2.5 mo; 25g) housed 5 per cage, with *ad libitum* access to food and water and a 12/12 hr light/dark cycle. Animals were acclimated to the facility for at least one week before handling. Procedures were approved by UC Berkeley and UC Davis animal care committees.

Rats were stressed by restraint in plastic decapicone bags for 3 hours each day for 1 week (stress n = 6; unstressed controls, n = 5). For cort studies, rats received 7 daily subcutaneous (*s.c.*) injections of cort (40mg/kg) or vehicle oil, a dose that reciprocates many

aspects of stress treatments[95–97], and were sacrificed on day 7 (vehicle, n=6; cort, n=6) or on day 14 (vehicle, n=7; cort, n=6). Intraperitoneal (*i.p.*) injections of BrdU (200mg/kg) were given on the first 3 days of stress or cort treatments.

Nestin-Cre ER^{T2} / RosaYFP mice[101] were given tamoxifen injections (180 mg/kg, *i.p.*) twice per day for 5 days to induce maximal recombination. 15 days later, mice were injected with cort (25 mg/kg, *s.c.*, n=8) or vehicle (n=8) for 10 days, and then sacrificed 7 days after the end of cort treatment. BrdU (50 mg/kg, *i.p.*) was injected twice per day on the first 3 days of cort treatment. At endpoints, animals were transcardially perfused with saline followed by cold 4% PFA.

Histology. Brains were post-fixed in 4% PFA, cryoprotected and cryosectioned (40µm coronal slices) through the dentate gyrus. Immunohistochemistry was performed with rat anti-BrdU (1:500; Accurate, Westbury, NY) and mouse anti-TuJ1 (1:5000; Covance), mouse anti-RIP (1:1000; Millipore, Billerica MA), or rat anti-MBP (1:100; Abcam) for 48h at 4°C; or rabbit anti-GST-π (1:5000; MBL, Woburn MA) and goat anti-GFP (targeting YFP; 1:500, Rockland Immunochemicals, Gilbertsville PA) for 18h at 4°C. Secondary incubations were FITC-conjugated donkey anti-mouse (1:500, Jackson) for 2 hours, and biotinylated anti-rat (1:500; Jackson) for 2 hours followed by Streptavidin-Alexa 568 (1:1000; Invitrogen) for 1 hour. For Olig1 labeling, sections were immunostained with rabbit Olig1 (1:5000, from Charles Stiles, Harvard University) and anti-rabbit Cy3 or anti-rabbit Cy5 (1:500, Jackson). For combined BrdU and MBP labeling, sections were incubated in mouse anti-BrdU (1:500, BD) and rat anti-MBP (1:100, Abcam), followed by incubation with FITC donkey anti-rat (1:500, Jackson) and then biotinylated anti-mouse (1:500, Jackson), and visualized with Streptavidin-Alexa 568 (1:1000, Jackson). Sections were coverslipped with DABCO anti-fading medium in TBS.

BrdU-labeled cells were visualized using a Zeiss 510 AxioImager confocal laser-scanning microscope with LSM 510 software. At least 25 BrdU-labeled cells were examined per rat, and co-labeling with cell fate markers was quantified. Confocal and z-stacked images were used in coordination to determine the percentage of BrdU+ cells co-labeling with cell fate markers. For cytoplasmic markers, fluorescence signals surrounding BrdU+ nuclei were considered positive. BrdU counts were quantified as total BrdU cells per tissue section, or as density measurements, in which the areas of the GCL and hilus were measured using Metamorph and multiplied by section thickness (40µm). Total BrdU-labeled cells within each ROI were then normalized to the total volume per animal. For transgenic lineage tracing, all YFP positive cells were examined in every sixth serial section throughout the mouse dentate gyrus (40µm section thickness), and scored for co-localization with GST-π.

Statistical analyses. Means and SEMs were determined for the above variables. For statistical comparisons, these values were subjected to unpaired two-tailed Students t-tests or one-way ANOVA. P-values ≤ 0.05 were considered significant.

2.5 Figures

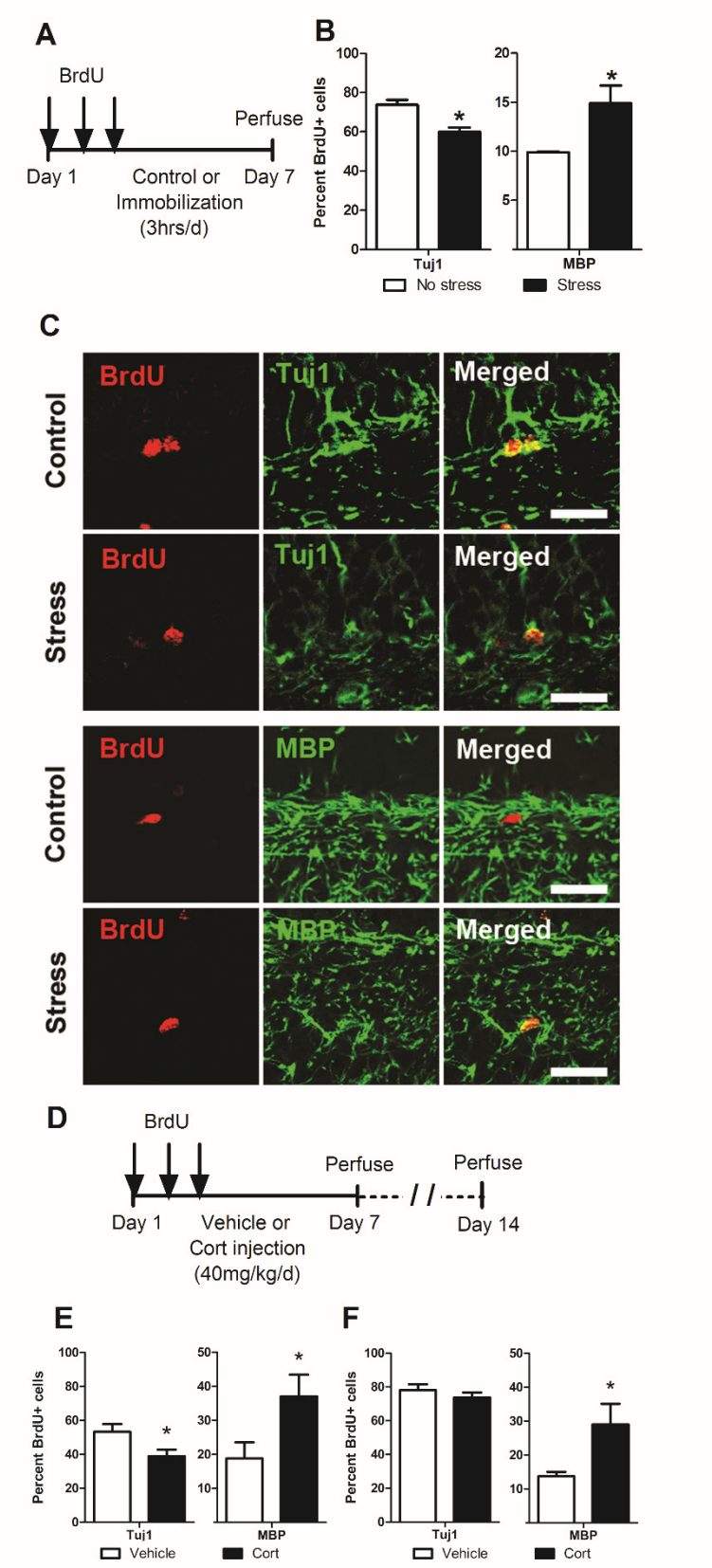

Figure 2.1. Immobilization stress or cort injections increase hippocampal oligodendrogenesis. (a) BrdU-injected adult male rats were subjected to either 1 week of daily immobilization stress or no stress (n=5 no stress control, n=6 stress). (b) IHC analysis of cell fate, quantified as the percentage of BrdU positive cells that co-label as neurons (Tuj1) or oligodendrocytes (MBP) shows that stress decreases neurogenesis and increases oligodendrogenesis. (c) Representative images of confocal analysis represent cells identified as positive for co-localization of BrdU and Tuj1 or MBP; scale bar=10 µM. (d) BrdU-injected adult male rats received daily cort or vehicle injections for 1 week and were perfused on day 7 (n=6 vehicle injected, n=6 cort injected) or day 14 (n=7 vehicle injected, n=6 cort injected). (e) IHC analysis of cell fate at day 7 shows that exposure to stress hormones (cort) decreases neurogenesis and increases oligodendrogenesis. (f) IHC analysis of cell fate at day 14 (1 week after recovery from cort treatment) shows that while neurogenesis is restored to control levels, the effects of increased oligodendrogenesis persist in cort injected animals. *$p < 0.05$ (mean ± SEM).

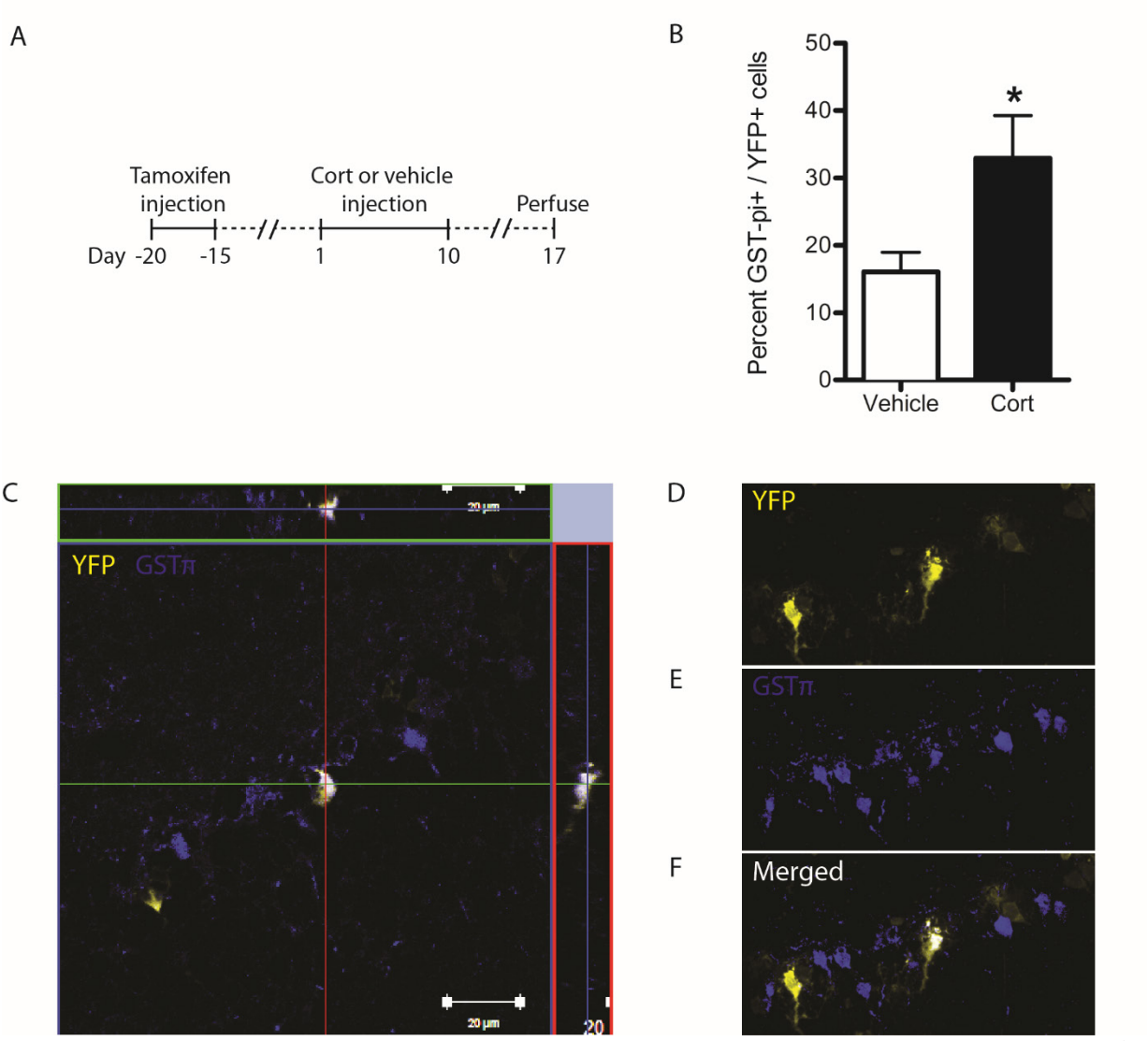

Figure 2.2. Cort increases hippocampal oligodendrogenesis from nestin lineage NSCs in vivo. (a) Nestin-Cre ER^{T2} / RosaYFP mice were injected with tamoxifen to induce YFP reporter gene expression, injected with cort or vehicle for 10 days, and perfused 7 days later for IHC analysis. (b) Cort injection increased the percentage of YFP-labeled cells that co-labeled with the oligodendrocytic marker GST-π; n=8. (c) Representative image of an orthogonal slice of a YFP+/GST-π+ co-labeled cell as well as 3D reconstruction of an image stack of the same cell (d-f). *$p < 0.05$ (mean ± SEM).

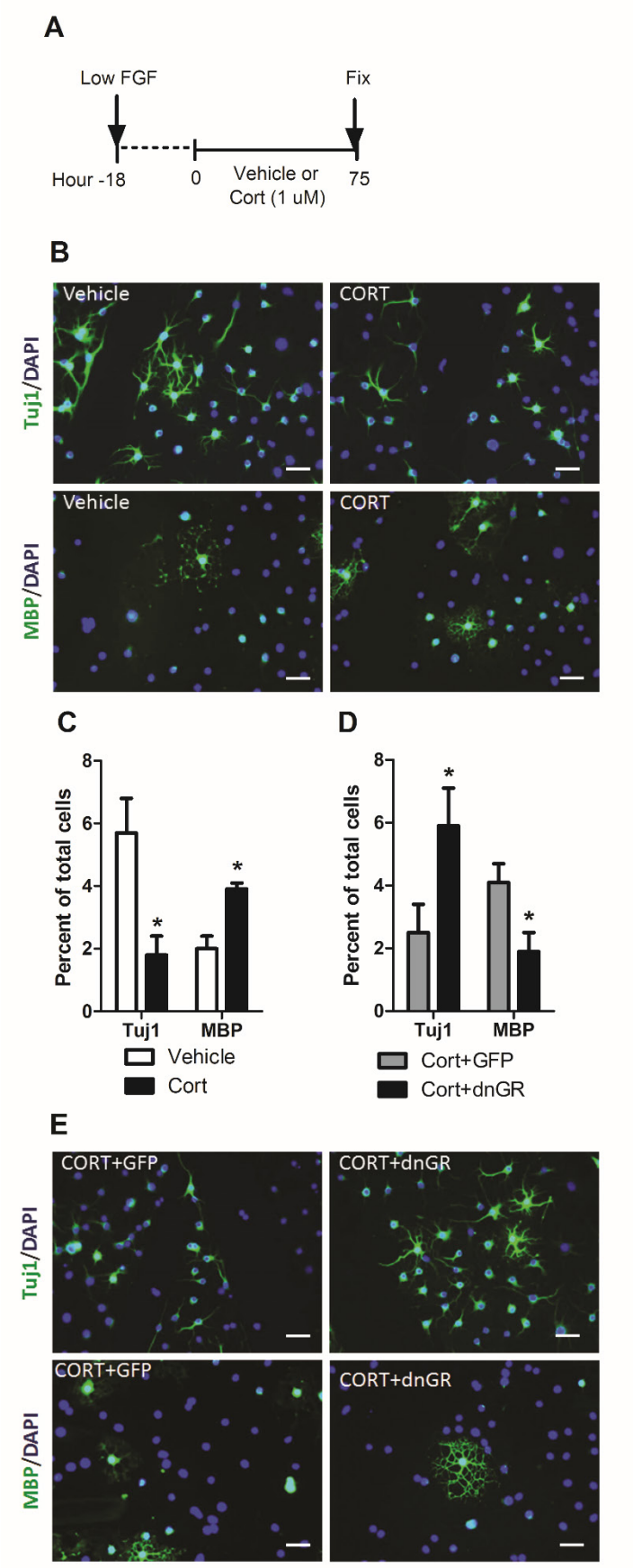

Figure 2.3. Cort treatment increases the oligodendrogenic potential of NSCs. (a) NSC cultures were treated with cort or vehicle for 75 h. (b) Representative images of ICC analysis, scale bar = 50 µm. (c) ICC analysis of cell fate, quantified as total number of cells labeling as neurons (Tuj1) or oligodendrocytes (MBP). (d, e) ICC analysis of cell fate of NSCs transfected with dnGR or GFP (control) and treated with cort. *$p < 0.05$ (mean ± SEM); n≥3.

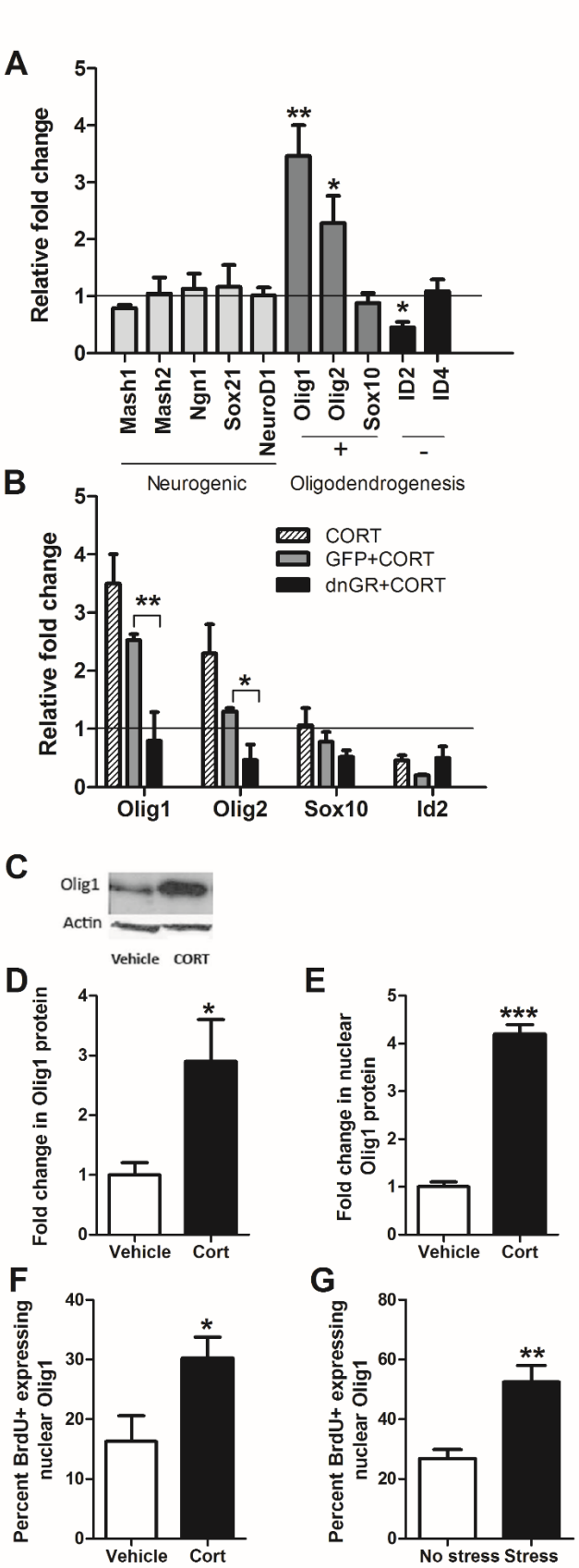

Figure 2.4. Cort treatment induces a pro-oligodendrogenic transcriptional program in NSCs. (a) Fold change in mRNA expression of genes regulating neurogenic and oligodendrogenic fate in cort-treated NSCs, relative to vehicle-treated controls, including genes that promote (+) and inhibit (-) oligodendrogenesis. (b) Fold change in mRNA expression of oligodendrogenic regulatory genes in NSCs transfected with dnGR or GFP (control) vectors, or no vector, and treated with cort, relative to vehicle-treated controls; n≥3. (c) Representative image of Western blot for Olig1 protein in NSCs treated with cort or vehicle. (d) Densitometric analysis of Western blot for total protein fraction and (e) the isolated nuclear fraction of treated NSCs; n=3. (f) IHC analysis of the percent of BrdU positive cells expressing nuclear-localized Olig1 in the hilus of adult rats after 1 week of daily cort injections; n=6 vehicle injected, n=6 cort injected (g) or after 1 week of daily immobilization stress; n=5 no stress control, n=6 stress. *p < 0.05, **p<0.005, ***p < 0.0001 (mean ± SEM).

Chapter 3. The role of age-related breakdown of the blood-brain barrier and TGF-beta signaling in cognitive impairment

Adapted from: Senatorov VV Jr*, Friedman AR*, Milikovsky DZ, Ofer J, Saar-Ashkenazy R, Charbash A, Jahan N, Chin G, Mihaly E, Lin JM, Ramsay HJ, Moghbel A, Preininger MK, Eddings CR, Harrison HV, Veksler R, Becker A, Hart B, Rogawski MA, Dillin A, Friedman A, and Kaufer D, 2018. Blood-brain barrier breakdown during aging causes chronic but reversible neural dysfunction via TGF-beta signaling. Manuscript in preparation. *Equal contribution.

3.1 Introduction

Across mammalian species, old age is associated with a decline in cognitive function, which is thought to result from underlying hallmarks of neural aging including synaptic loss and other impairments in neural function[44,45]. Most, if not all, aged humans experience these relatively subtle deficits, while some experience much more severe, neurodegenerative decline such as Alzheimer's disease and related dementias (ADRD). It is currently unknown whether the "natural" cognitive decline that is widespread and apparently normal in aging is functionally distinct from severe ADRD, or alternatively, whether the early stages of mild cognitive decline may be the first step on a pathway leading to ADRD. We also have a poor understanding of what first triggers the biological transition from a youthful, healthy brain into the mild cognitive impairment of aging. However, it is notable that age-related cognitive impairments share a remarkably similar set of symptomatic outcomes to head injury: both involve initial BBB disruption followed by secondary neural dysfunction and cognitive impairment[141–143]. On the etiological levels of underlying molecular and electrophysiological mechanisms, the connections may be even stronger. There is evidence that the earliest stages of mild cognitive impairment, which can precede significant Aβ plaque accumulation and neurodegeneration in AD, may be caused by excitation / inhibition imbalance and hyperexcitability in neural circuits[144–147]. In studies focused on rodent models for head injury and post-traumatic epilepsy, exogenous application of albumin to the brain triggers TGFβ signaling, which directly remodels neural circuits to cause hyperexcitability[64,66]. We reasoned that in aging, BBB dysfunction may play a similar role, in which TGFβ signaling causes neural dysfunction and hyperexcitability that could account for a range of age-related symptoms including cognitive impairment.

To test this hypothesis, we characterized the time course and progression of BBB breakdown over the lifespan of aging mice, as well as the relationship between BBB dysfunction and electrophysiological hallmarks of neural dysfunction and cognitive decline. Furthermore, we tested whether infusion of albumin into the brains of otherwise healthy, young mice was sufficient to cause neural and cognitive dysfunction similar to that of aged mice. Finally, we used genetic intervention to inhibit TGFβ signaling in mice, to assess whether TGFβ signaling is a mechanistic cause of cognitive outcomes that follow albumin infusion.

3.2 Results

Aging mice experience progressive BBB dysfunction that is associated with TGFβ signaling and aberrant neural activity

Rodent models offer an ideal system to investigate the underlying mechanisms and outcomes of BBB dysfunction in age-related disease; however, there have been very few studies investigating BBB status in naturally aging rodents[148]. Thus, our initial analysis focused on comprehensively characterizing the time course of BBB dysfunction during natural aging, to establish the temporal relationship between vascular leakiness and the onset of age-related neural dysfunction. Given the putative role of astrocytes in BBB-induced TGFβ signaling, we used a transgenic mouse line labeling astrocytes via the Aldh1L1 promoter (Rep-Aldh1L1)[149]. This pan-astrocytic promoter labels a greater number and variety of astrocytes than traditional approaches using markers such as GFAP, enabling more comprehensive analysis of the aging astrocyte population[149,150]. We found that expression of this marker was stable and did not change across the lifespan. Using Rep-Aldh1L1 mice, we quantified albumin extravasation and astrocytic uptake of albumin at time points across the mouse lifespan, with analysis focused on the hippocampus, given its essential roles in age-related memory decline and implication as one of the earliest areas of BBB decline in aging humans[59]. Albumin was effectively absent from the hippocampus of young mice but appeared in the aging hippocampus starting as early as 7 months ("middle age") with levels increasing in aging up to two years, near the end of life (Figure 3.1A-B). These findings in aged mice correspond to previous reports that albumin uptake occurs primarily in astrocytes in vitro and following experimentally-induced BBB disruption in vivo[64,67]. In these studies, albumin endocytosis into astrocytes is mediated by direct binding to the TGFβ receptor II (TGFβR) subunit, which activates the TGFβR ALK5[64,66] and induces the phosphorylation of Smad2 (pSmad2), the primary signal transduction protein that initiates the ALK5-TGFβ signaling cascade. Further, this activation also increases the production of TGFβ1 in astrocytes[66] and activation of latent TGFβ1 protein from extra-cellular matrix[71], yielding an increase in the canonical ligand of TGFβR and therefore amplification of the TGFβ cascade. Hence, we focused our subsequent analysis on astrocytes and investigated the relationship between albumin uptake and TGFβ signaling in the aged mouse brain. Concurrent with the time course of albumin extravasation, aging mice showed a progressive increase in the levels of pSmad2, co-localized with albumin-positive astrocytes (Figure 3.1C-E). Activation of the TGFβ pathway was further quantified via Western blot showing increased levels of pSmad2 in the hippocampus of old mice, compared to young, as well as increases in active TGFβ1 (Figure 3.1F).

Based on the mechanistic insights from rodent head injury models, in which TGFβ signaling induced by BBB dysfunction causes reorganization of neural networks and hyperexcitability, we predicted that TGFβ signaling may similarly account for abnormal excitability associated with aging in both rodents and humans[74,144,145,147]. Thus, we performed telemetric electrocorticography (ECoG) to search for evidence of network dysfunction in aging mice. In comparing recordings taken from young and old mice over a 5 day period in the home cage, we found that aged mice showed an increase in the relative power of slow wave activity (<5 Hz), similar to EEG slowing described in human dementia patients[151–153], which is thought to reflect dysfunctional neural networks. Detailed analysis of this aberrant ECoG signal revealed that the slow-wave activity was not continuous, but rather manifested in discrete, transient paroxysmal slow wave episodes (SWEs, median frequency < 5 Hz; Figure 3.1G). Strikingly, approximately half of the aged mice showed a highly elevated number of SWEs with thousands

of paroxysmal events per day. Using an unbiased Gaussian mixed model clustering approach, we found that the aged mice stratified into two statistically distinct clusters, one with high number of SWE per day (h-SWE) and one with low number of SWE (l-SWE) per day (Figure 3.1H). In contrast, the young mice group formed in a single cluster, with a low average number of SWEs per day, similar to the l-SWE aged group. The duration of SWEs was not different across groups, indicating that the increases in the h-SWE group were driven by an increase in the number, not in duration, of SWEs. Based on this clustering, we analyzed relative power across the broader ECoG spectrum to compare activity across the three subgroups (young, old l-SWE, and old h-SWE), further showing that old h-SWE have increased low-frequency power (delta band) and corresponding decreases in higher frequency activity (theta – beta), whereas the spectral power of the old l-SWE mice did not differ from that of young mice (Figure 3.1I). These results suggest that ECoG analysis of discrete SWEs can provide a "fingerprint" characterizing a sub-population of aged mice that are affected by recurrent episodes of transient, hyper-synchronized pathological neural network activity. In turn, this may provide a useful biomarker to assess whether therapeutic interventions are effective in restoring healthy neural network activity.

Infusion of albumin into the brains of young rodents is sufficient to reproduce the neurological phenotype of aged mice.

We used two experimental manipulations to test the necessity and sufficiency of TGFβ signaling in causing neural and cognitive dysfunction associated with BBB breakdown. Firstly, we generated a transgenic mouse line (aTGFβR2/KO) expressing inducible Cre recombinase under the astrocyte-specific GLAST promoter. This enables targeted knock-out of the floxed TGFβR with temporal precision, specifically in astrocytes (Figure 3.2A), allowing us to interrogate the role of astrocytic TGFβ signaling in mediating pathological outcomes. Treatment with tamoxifen (tam) efficiently induced recombination in approximately 40% of hippocampal astrocytes but not in neurons (Figure 3.2B-C), and significantly reduced levels of TGFβR (Figure 3.2D). To test if TGFβ signaling, as induced by BBB dysfunction, is sufficient to cause the aberrant neural activity (apart from other potential aging factors), we infused albumin (iAlb) or artificial cerebrospinal fluid (aCSF) into the brain ventricles (icv) of healthy, young adult rats and mice via osmotic mini-pump for 7 days, thus mimicking the slow diffusion of this serum protein into the brain as occurs after BBB breakdown (Figure 3.2E). Following icv infusion, the exogenous albumin diffused readily into the ipsilateral hippocampus, and was taken up by astrocytes within 48 hours of infusion (Figure 3.2F), similar to uptake seen in previous studies (Braganza et al., 2012; Weissberg et al., 2015). Albumin infusion induced an increase in the levels of GFAP and pSmad2 protein in the rat hippocampus (Figure 3.2G-H), similar to levels of increase observed in old mice.

We then examined if infusion of albumin is sufficient to induce aberrant neural ECoG activity in young rodents, similar to the activity observed in aging. Recordings of ECoG activity in iAlb rats showed an increase in slow-wave power, including an increase in slow-wave delta and decrease in alpha and beta power (Figure 3.3A), mirroring the aberrant activity seen in old h-SWE mice. Like old h-SWE mice, slow-wave activity in iAlb rats was characterized by SWEs, which were significantly increased in the ipsilateral hemisphere receiving iAlb, compared to

contralateral hemisphere (Figure 3.3B-D). We next used young aTGFβR KO to test the necessity and sufficiency of TGFβ signaling in cognitive impairment. Following induction with tamoxifen, we implanted young aTGFβR KO and control mice with iAlb (or aCSF control) osmotic pumps for one week. One month later, we tested mice in the Morris water maze (MWM) spatial memory task. In mice that were heterozygous for floxed TGFβR2 ($Tgfbr2^{fl/+}$), and hence had intact TGFβ signaling, iAlb infusion caused significantly impaired memory performance over 9 days of MWM training (Figure 3.3E). Furthermore, aTGFβR KO partially rescued this deficit, with iAlb $Tgfbr2^{fl/fl}$ KO mice showing an intermediate learning curve between the performance levels of iAlb and aCSF $Tgfbr2^{fl/+}$ mice.

Together, these data show that experimental induction of TGFβ signaling by albumin infusion is sufficient to confer an "aged" phenotype in young mice, including gliosis, aberrant neural activity, and cognitive impairment. Furthermore, selective knock out of TGFβR expression in astrocytes in young mice rescues albumin-induced cognitive impairment, indicating that activation of astrocytic TGFβR is required to attain the aged brain cognitive phenotype.

3.3 Discussion

Aging is often accompanied by cognitive decline, even in the absence of dementia or measurable neurodegeneration[44,45]. Unlike transgenic models for artificially inducing age-like disease, our investigations focused on naturally aging mice, allowing us to observe the relative sequence of biological changes associated with brain aging. We found that BBB dysfunction and consequent albumin extravasation appears as early as middle age, placing it among the earliest known hallmarks of the aging brain. Consistent with our findings, changes in the expression of key neurotransmission proteins have been widely observed in humans and other mammals as one of the first signs of neurological aging[44,45,155,156], and these changes in neurotransmission are associated with hippocampal hyperexcitability that is thought to be one of the earliest events in the progression of mild cognitive impairment[146,147,157]. However, the regulatory pathways that trigger or control these changes are unknown. Our research group has found that microvascular BBB dysfunction allows serum albumin to enter into the brain, where it binds to astrocytic TGFβR and leads to abnormally increased TGFβ signaling[64,67]. Activation of this signaling cascade in turn causes transcriptional down-regulation of GABAergic and glutamatergic neurotransmission proteins[64]. This suggests a straight-forward mechanism through which BBB dysfunction may cause dysfunction in the aging brain, by altering the levels of the foundational proteins controlling neural signaling.

In line with this putative mechanism, we used telemetric ECoG to directly record abnormal neural network activity during aging. We found slowing of EEG/ECoG activity, consistent with other reports in the context of aging[151–153]. We further showed that this slowing of activity in mice is characterized as discrete, paroxysmal transient events (SWEs), occuring frequently and spontaneously against a backdrop of "normal" ECoG activity. These SWEs are reminiscent of paroxysmal events that appear during epileptogenic network reorganization (prior to the onset of spontaneous seizures) in rodent models of epilepsy[158], and of aberrant EEG activity that has been observed and diagnosed as subclinical epileptiform activity and/or "silent seizures" in aging human patients[159–161]. These data support the suggestion, proposed

elsewhere[141–143], that there may be common mechanistic links between age-related dementia and epilepsy, which has a remarkably high incidence in the elderly[162–164]. We emphasize that changes in neurotransmission machinery, triggered by BBB dysfunction and TGFβ signaling, provide a simple, parsimonious model for how this dysfunction may arise in aging. Indeed, we found that simply infusing albumin into the otherwise healthy, young mouse brain was sufficient to induce aberrant SWEs, suggesting that BBB leakiness and specifically albumin activation of TGFβ signaling can directly cause perturbation of neural networks. Furthermore, infusion of albumin was sufficient to cause cognitive impairment, suggesting aberrant activity observed on the level of ECoG may is not merely an artifact or subtle perturbation, but rather that it may pertain to a genuine functional deficit that impairs the cognitive capacity of the mouse.

3.4 Methods

Immunostaining. Mice were anesthetized with Euthasol euthanasia solution and transcardially perfused with ice cold heparinized physiological saline (10 units heparin/mL physiological saline) followed by 4% paraformaldehyde (PFA, Fisher Scientific #AC416785000) in 0.1 M phosphate buffered saline (PBS). Brains were removed, post-fixed in 4% PFA for 24 hours at 4° C, and cryoprotected in 30% sucrose in PBS. Brains were then embedded in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA), frozen, and sliced on a cryostat into 20 µm coronal sections, mounted on slides. All samples were immunostained under the following protocol. Slides were treated for antigen retrieval (for human, 5 min incubation at 100° C in sodium citrate buffer, pH 6.0); for mouse, 15 min incubation at 65 °C in Tris-EDTA buffer (10mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0), then incubated in blocking solution (5% Normal Donkey Serum in 0.1% Triton X-100/TBS) for 1 hour at room temperature. Samples were then stained with primary antibody at 4° C, followed by fluorescent-conjugated secondary antibody for 1 hour at room temperature, and then incubated with DAPI (900 nM; Sigma-Aldrich) to label nuclei. Primary antibodies were rabbit anti-phosphorylated Smad2 (Millipore AB3849-I, 1:500), chicken anti-Albumin (Abcam ab106582, 1:500), and goat anti-GFAP (Abcam ab53554, 1:1000). Secondary antibodies were anti-rabbit Alexa Fluor 568, anti-chicken Alexa Fluor 647, anti-goat Alexa Fluor 488, and anti-goat Alexa Fluor 647 (Abcam ab150131, 1:500). All antibodies dilutions were in blocking solution. For tissue from aged mice, slide-mounted brain sections for treated with TrueBlack Lipofuscin Autofluorescence Quencher (Biotium #23007) before coverslip mounting.

Microscopy. Images were acquired at 20X or 40X magnification using a Zeiss Axio Observer Research microscope (Carl Zeiss AG) and Metamorph software (version 7.7.7.0) and analyzed using ImageJ software (NIH). Imaging was performed in at least 3 hippocampal sections per mouse. Cell counts for each individual marker, and colabeling, were calculated manually by an observer blind to experimental conditions and normalized to the total number of DAPI-positive cells. For each subject, counts from each sampling area were averaged.

Western Blot. Mouse or rat hippocampal hippocampal tissue was homogenized and protein lysates were extracted using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) including a protease (Calbiochem #539134) and phosphatase inhibitor cocktail (Roche PhoStop Ref: 4906845001). Protein samples were run

under reducing conditions. 20 µg of protein lysate was mixed with Laemmli buffer (Bio-Rad #161-0737), containing 5% 2-mercaptoethanol (Sigma M6250), and fractionated by SDS-PAGE using the Mini-PROTEAN Tetra System and pre-cast TGX™ Gels (Bio-Rad #456-1096); Following separation, samples were transferred to a nitrocellulose membrane (0.45 µm, Bio-Rad #1620115). Membranes were blocked for 1 hr at room temperature with 5% non-fat dry milk (Apex #20-241) in TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH 8.0), and incubated overnight at 4 °C with primary antibody. Membranes were then washed 3x10 min with TBST and incubated with secondary antibodies for 1 hr at room temperature. Membranes were washed with TBST 3x10min and visualized using chemiluminescence SuperSignal West Dura Extended Substrate (ThermoFisher Scientific #34075), and Bio-Rad Chemidoc system with Bio-Rad Image Lab software (version 4.0.1). Densitometry analysis was done using Image J (NIH). The following primary and secondary antibodies were used: rabbit anti-β-Actin (1:2000, Cell Signaling #4970), rabbit anti-GAPDH (1:2000, Cell Signaling #2118), rabbit anti-TGFβR2 (1:1500, Abcam ab186838), rabbit anti-phosphorylated Smad2 (1:1000, Millipore AB3849-I), rabbit anti-phosphorylated Smad2 (1:1000, Millipore AB3849), chicken anti-Albumin (1:1500, Abcam ab106582), goat anti-GFAP (1:1000, Abcam ab53554), anti-goat HRP (1:1000, R&D systems HAF109), anti-mouse HRP (1:2000, Cell Signaling #7076), anti-rabbit HRP (1:2000, Cell Signaling #4970), and anti-chicken HRP (1:2000, ThermoFisher Scientific A16054)**.**

Osmotic pump implants. For mice, pumps were implanted as previously described[165]. Briefly, surgery was performed on adult male mice under isofluorane anesthesia (2%). Using a stereotaxic frame, a hole was drilled through the skull at 0.5 mm posterior, 1 mm lateral to bregma, and a cannula was placed into the right lateral cerebral ventricle, fixed with surgical glue. Cannulas (Brain infusion kit BIK 3, #0008851, Alzet, Cupertino, CA) were attached to micro-osmotic pumps (Model 2001, ALZET) filled with 200 µL of either 0.4 mM bovine serum albumin (Alb; Sigma-Aldrich) solution or artificial cerebrospinal fluid (aCSF) as previously described[166], and implanted subcutaneously in the right flank. In a subset of animals, 10% of the Alb was replaced with Alexa Fluor 647 conjugated BSA (2.68 g/L; ThermoFisher Scientific A34785). Pumps infused at a rate of 1.0 µL/hr for the duration described in each experiment. For rats, 10-week-old male Wistar rats were used, and surgeries were performed the same way, using the following coordinates: -1 mm posterior and 1.5 mm lateral to bregma. For rats, albumin was used at a concentration of 0.2 mM and infused for 7 days at a rate of 10 µL/hr via larger micro-osmotic pumps (Model 2ML1, Alzet).

ECoG. ECoG was recorded as previously reported[158] from 9- to 12-wk-old Wistar male rats implanted with osmotic pumps, and from young (3 months, n = 5) and old (18-22 months, n = 20) mice. In brief, under stereotaxic surgery and 2% isoflurane anesthesia, two screw electrodes were implanted in each hemisphere (rat coordinates: 4.8 mm posterior, 2.7 mm anterior, and 2.2 mm lateral; mouse coordinates: 0.5 and 3.5 mm posterior and 1 mm lateral, all relative to bregma). A wireless transmitter (Data Science International, Saint Paul, MN, US) was placed in a dorsal subcutaneous pocket, and leads connected to the screws. Connections were isolated and fixed by bone cement such that one ECoG channel was associated with each hemisphere. For rat surgeries, a cannula (-1 mm caudal, 1.5 lateral, 4 mm depth) and osmotic pump (infusing at 2.4 µl/h) were also implanted[66]. Animals were treated with post-operative buprenorphine (0.1 mg/Kg) and allowed to recover for at least 72 hrs prior to recording. Continuous, bichannel ECoG (sampling rate, 500Hz) was recorded wirelessly from freely

roaming animals in the home cage for the duration of experiments described. Rats were recorded during the first and the fourth week following surgery. Mice were recorded for 5 days, starting 1 week after surgery, and administered IPW (20 mg/kg) on days 6-10 of recording. To detect SWE, ECoG signals were buffered into 2 sec long epochs with an overlap of 1 sec. Fast Fourier Transform (FFT) was applied and the frequency of median power was extracted for each epoch. Thus, an event was considered as a SWE if its frequency of median power was less than 5 Hz for 10 consecutive seconds or more. FFT was also applied for the entire recording period to analyze relative power across the frequency spectrum of 1-20 Hz.

Animal care and transgenic mice. All animal procedures were approved by the institutional animal care committees. Animals were housed with a 12:12 light:dark cycle with food and water available ad libitum. Aldh1L1-eGFP mice were bred from STOCK Tg(Aldh1l1-EGFP)OFC789Gsat/Mmucd (identification number 011015-UCD), purchased from the Mutant Mouse Regional Resource Center. These FVB/N mice were crossed to a C57BL/6 genetic background. The resulting strain exhibits constitutive astrocytic expression of eGFP protein under the astrocytic promoter Aldehyde Dehydrogenase 1 Family, Member L1 (Aldh1L1).

Triple transgenic aTGFβR/KO mice were bred from strains purchased from the Jackson Laboratory to generate mice that express CreERT under the astrocytic promoter glial high affinity glutamate transporter (GLAST), with a floxed exon 4 of TGF-βR2 ($tgfbr2^{fl}$), and a transgenic LacZ reporter gene inhibited by a floxed neomycin cassette. Tamoxifen induction thus induces activation of astrocytic CreERT resulting in a null TGFβR2 allele (tgfbr2null) and LacZ expression ($R26R^{-/-}$). The parental strain STOCK Tg(Slc1a3-cre/ERT)1Nat/J mice were outcrossed with B6;129-Tgfbr2tm1Karl/J and B6.129S4-Gt(ROSA)26Sortm1Sor/J mice to produce males, while B6;129-Tgfbr2tm1Karl/J and B6.129S4-Gt(ROSA)26Sortm1Sor/J mice were outcrossed to produce females. The resulting GLAST-CreERT; $tgfbr2^{fl/+}$ males were bred with tgfbr2fl/+; R26R-/- and $tgfbr2^{fl/fl}$; $R26R^{-/-}$ females to produce triple transgenic offspring. Subsequent generations were incrossed to produce experimental triple transgenic mice of genotypes GLAST-CreERT; $tgfbr2^{fl/fl}$; $R26R^{-/-}$, GLAST-CreERT; $tgfbr2^{fl/+}$; $R26R^{-/-}$, GLAST-CreERT; $tgfbr2^{fl/fl}$; $R26R^{-/+}$, and GLAST-CreERT; $tgfbr2^{fl/+}$; $R26R^{-/-}$. All mice were genotyped via PCR analysis of tissue biopsy samples.

The inducible Cre/lox system was activated by 5 days of tamoxifen injection (Sigma-Aldrich, 160 mg/kg dissolved in corn oil, i.p). Control GLAST-CreERT; $tgfbr2^{fl/+}$ heterozygotes received the same dosage of tamoxifen and control GLAST-CreERT; $tgfbr2^{fl/fl}$ mice received i.p. injection of corn oil vehicle at equivalent volumes. Mice were weighed daily to ensure accurate dosage.

Behavior assessments. Spatial memory was assessed in the Morris Water Maze (MWM) task[167]. For each trial, mice were placed at randomized starting locations in a MWM pool filled with opaque water (colored with non-toxic white acrylic paint), with visual cues placed on the pool walls. Mice were allowed to swim freely until locating a hidden platform under the surface of the water (or guided to the platform after 40 seconds of failed searching), and then left on the platform for 10 seconds prior to starting a new trial. Spatial learning was quantified by measuring latency to reach the platform, averaged from four trials per day, with training over 9 consecutive days of learning.

Statistics and data. Data collection and preparation was performed by researchers who were blind to experimental conditions, and the lead and corresponding authors were

responsible for experiment design, conducting statistical analysis, and unblinding the final results. All graphs are plotted showing mean and SE. Two sample comparisons were performed by student's t-test or Mann-Whitney test, and multiple group comparisons were conducted by ANOVA or Kruskal–Wallis test, followed by post-hoc testing to compare individual groups when a main effect was detected. Multiple correction comparisons were used as described in figure legends. Different subgroups that were observed in ECoG recordings were first classified by an unbiased Gaussian mixed model, and then inferential statistics were performed on the subsequent groups. For all inferential statistics, two-tailed tests were used and significance thresholds were set at $p<0.05$.

3.5 Figures

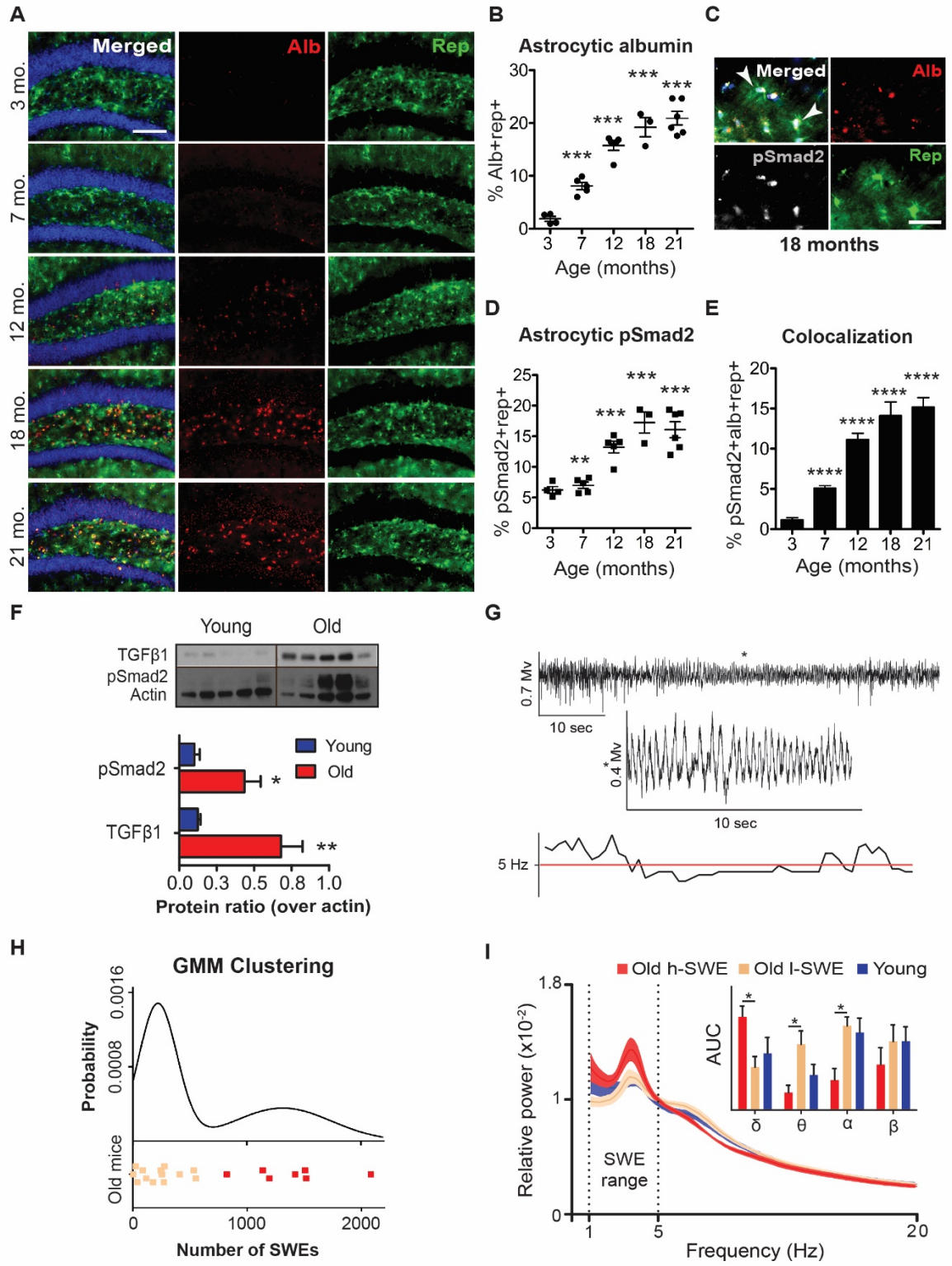

Figure 3.1. Progressive BBB dysfunction in aging mice is associated with elevated TGFβ signaling and aberrant network activity. (A) Immunofluorescent staining used to quantify progression of BBB decline in the aging mouse hippocampus at 3, 7, 12, 17, and 21 months old. Astrocytes are labeled by GFP expressed under the pan-astrocytic promoter Aldh1L1 (Rep-aldh1L1-eGFP), albumin is labeled by immunostaining, and cell nuclei by DAPI

(blue). Scale bar = 100 µm. (B) Albumin localized in astrocytes increases with age (ANOVA, $p < 0.0001$). (C) Co-localization of albumin and pSmad2 (arrows), visualized by immunostaining (Scale bar = 30 µm). Aging mice show an increase in the level of (D) pSmad2 colabeled with astrocytes (ANOVA, $p = <0.0001$), and (E) the percentage of astrocytes co-localizing with both albumin and pSmad2 (ANOVA, $p < 0.0001$). For all measures, groups were compared by Bonferroni post-hoc test. Sample sizes are n = 4 (3 mo); 5 (7 and 12 mo); 3 (18 mo); and 6 (21 mo). (F) Western blot analyzing TGFβ1 and pSmad2, outputs of the TGFβ signaling pathway, in hippocampus from young and old mice. Western blot densitometry shows that pSmad2 ($p=0.019$) and TGFβ ($p = 0.005$) protein levels were elevated in the aged mouse hippocampus (t-test with Bonferroni correction, $p=0.019$ and 0.005 respectively, n = 5). (G) A representative trace shows a SWE with slow-wave activity less than 5 Hz and a 10 second window of the slow wave event (marked with *). (H) Slow-wave episodes were quantified from continuous ECoG recordings in young and old mice, and GMM clustering was used to classify old mice into h-SWE and l-SWE subgroups. (I) Relative power was compared across the frequency spectrum from 1-20 Hz for all groups. Area under the curve (AUC) measures for each frequency band (δ, 1-5 Hz; θ, 6-8 Hz; α, 9-12 Hz; β, 13-20 Hz) showed that h-SWE old mice exhibited an increase in power in the slow-wave δ band and a decrease in θ and α (ANOVA, interaction $p < 0.01$, with Bonferroni posttest, n = 6 h-SWE, 6 l-SWE, 5 young).

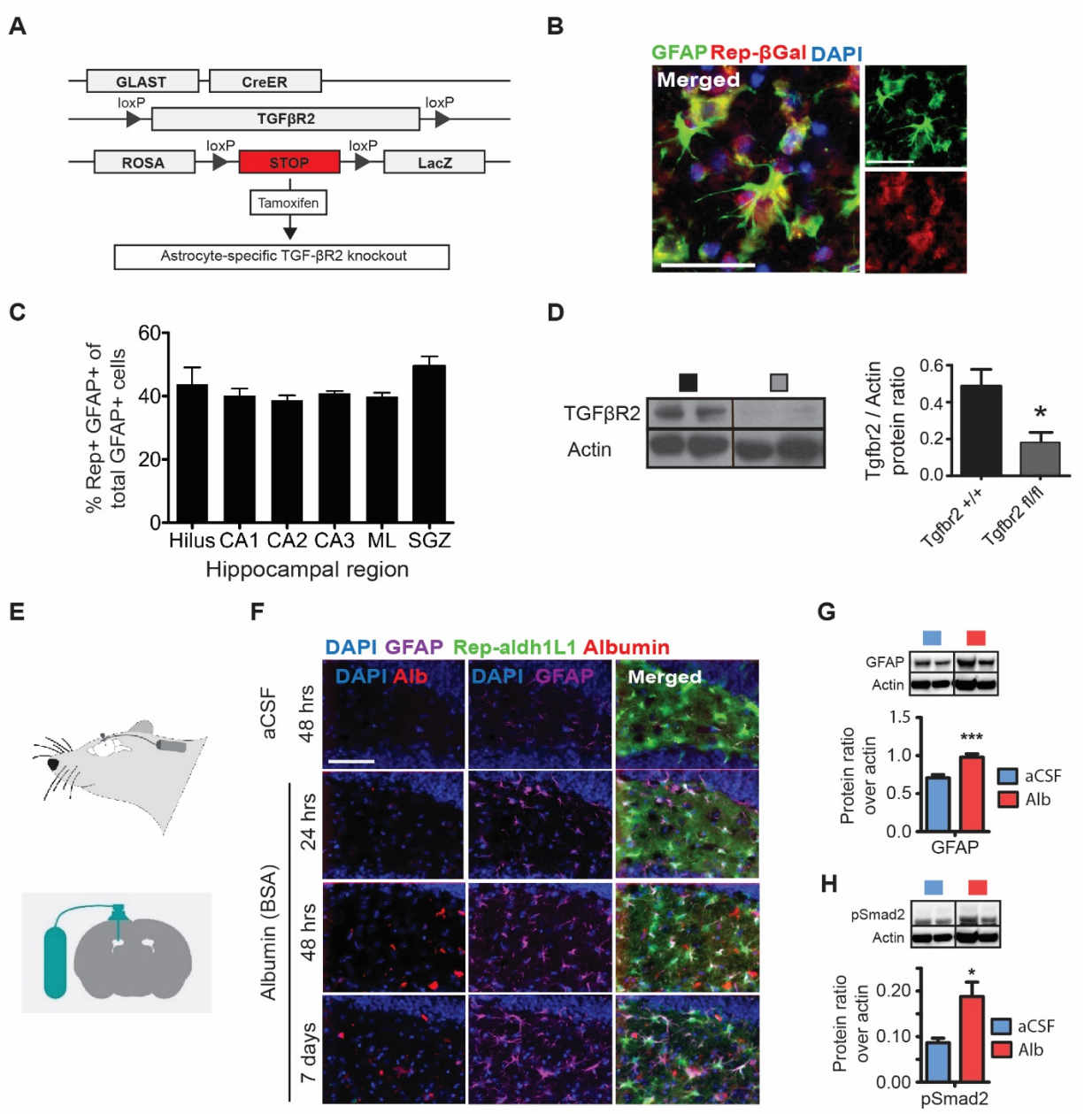

Figure 3.2. Knock-out of astrocytic TGFβ reverses neurological outcomes in aging mice. (A) Schematic of the transgenic aTGFβR KO system. The astrocytic GLAST promoter drives expression of Cre recombinase in astrocytes. Following induction with tamoxifen injection, activated Cre excises the TGFβR gene at inserted loxP sites. LacZ reporter expression provides a readout of Cre activity. (B) Representative immunofluorescent image from mouse hippocampus, showing reporter expression (Rep-βGal, red) in astrocytes (GFAP, green) following induction of the Cre recombinase system. (C) Reporter expression was present in approximately 50% of hippocampal astrocytes and was consistent throughout all hippocampal subregions (CA: cornu ammonis; ML: molecular layer; SGZ: subgranular zone). (D) Western blot with densitometry confirmed significant reduction in levels of TGFβR in the hippocampus following tamoxifen induction. (E) Diagrams showing mouse icv pump implants, with cannulas placed to infuse albumin into the brain ventricle. (F) Representative images from the dentate gyrus show albumin uptake in astrocytes at 48 hours and 7 days after pump implant. (G) Young adult rats were given icv albumin infusion (iAlb) for 7 days. iAlb increased protein levels of the activated astrocyte marker GFAP (t-test, $p = 0.004$, $n = 4$) and (H) pSmad2 ($p = 0.022$).

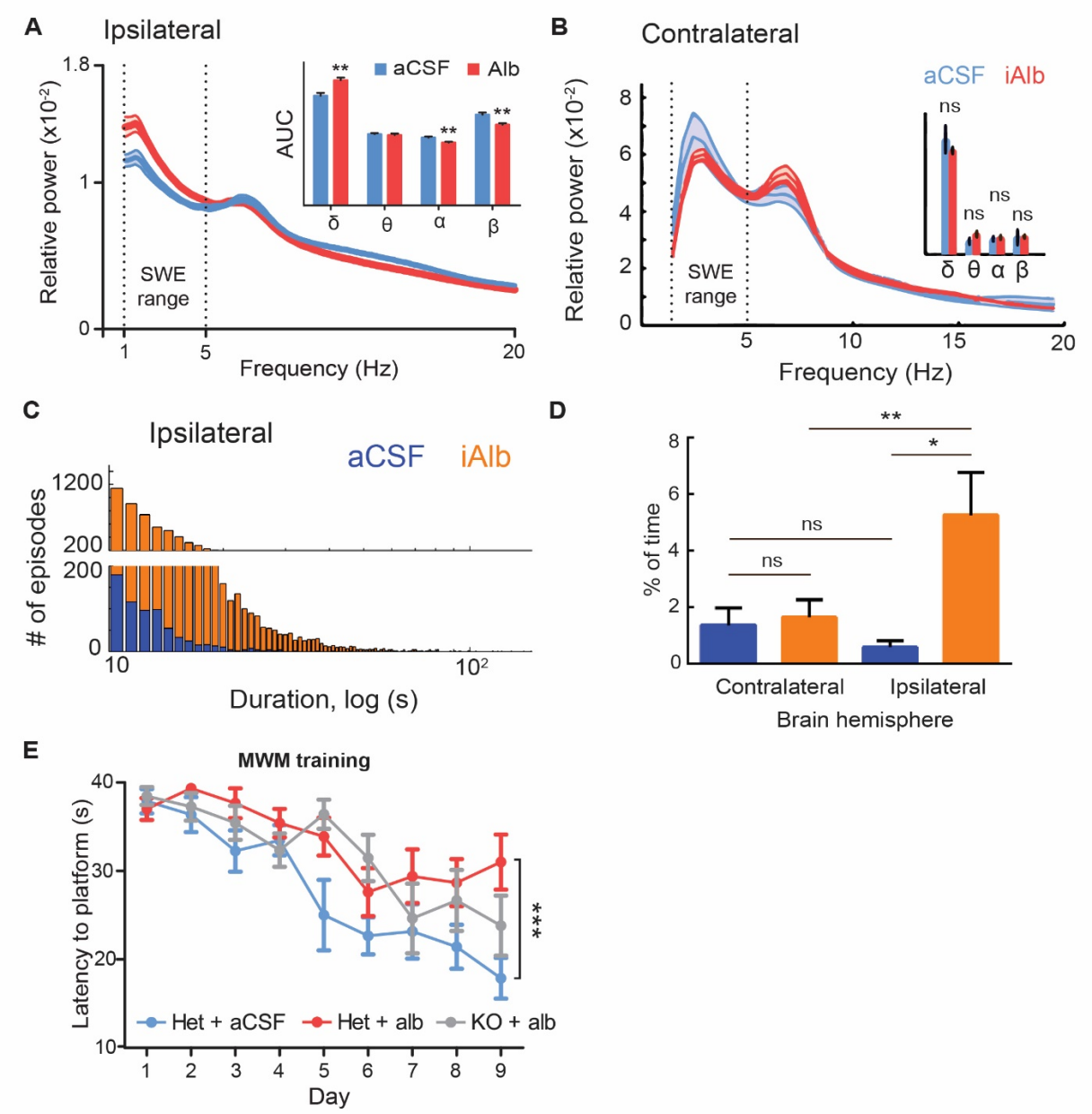

Figure 3.3. (A) ECoG activity was recorded from iAlb rats. AUC measures showed that albumin caused an increase in relative power in δ, with a corresponding decrease in α and β power (Mann-Whitney test, n = 5 aCSF, 8 alb). (B) No shift in the spectrum of ECoG activity was observed in recordings from contralateral hemisphere of iAlb rats. (C) Histogram showing number of SWEs recorded from ipsilateral cortex, binned by duration of SWEs. iAlb rats had significantly elevated number of SWEs compared to aCSF-infused controls. (D) Elevated SWEs were specific to ipsilateral cortex of iAlb rats, which showed SWEs occurring during nearly 6% of total recording time. In contrast, the contralateral hemisphere of iAlb rats, and both hemispheres of aCSF controls, had much lower percent of SWEs. (E) Following induction with tamoxifen, young aTGFβR KO and control mice were given iAlb surgery and then tested in the MWM task one month later. Compared to aCSF controls, iAlb Tgfbr2$^{fl/+}$ mice had significantly poorer learning performance, and KO (Tgfbr2$^{fl/fl}$) partially rescued performance following iAlb (2-way repeated measure ANOVA, main effect of learning over time $p < 0.0001$, main effect of group $p = 0.0093$, with Bonferroni posttest on day 9 showing significant differences between aCSF and iAlb Tgfbr2$^{fl/+}$, $p < 0.001$, but not between iAlb and KO. n = 9 aCSF Tgfbr2$^{fl/+}$, 10 iAlb Tgfbr2$^{fl/+}$, and 8 iAlb Tgfbr2$^{fl/fl}$). For all tests, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

Chapter 4. Interventions against TGFβ signaling to restore cognitive function in aging

Adapted from: Senatorov VV Jr*, Friedman AR*, Milikovsky DZ, Ofer J, Saar-Ashkenazy R, Charbash A, Jahan N, Chin G, Mihaly E, Lin JM, Ramsay HJ, Moghbel A, Preininger MK, Eddings CR, Harrison HV, Veksler R, Becker A, Hart B, Rogawski MA, Dillin A, Friedman A, and Kaufer D, 2018. Blood-brain barrier breakdown during aging causes chronic but reversible neural dysfunction via TGF-beta signaling. Manuscript in preparation. *Equal contribution.

4.1 Introduction

Our initial experiments established that BBB dysfunction occurs relatively early in aging and is tightly linked with astrocytic TGFβ signaling and the onset of neural dysfunction. Brain infusion experiments in young mice further showed that experimental induction of TGFβ signaling is sufficient to induce outcomes that recapitulate the pathology of aging. However, given the myriad of deficits and failures that occur across a wide range of biological systems in aging and senescence, it is uncertain whether BBB breakdown and TGFβ signaling play a key role in cognitive decline, or alternatively whether they may be an ancillary symptom of an otherwise ailing brain. Thus, from a translational therapeutic perspective, it is uncertain whether interventions against TGFβ signaling may have any efficacy in improving cognitive outcomes in aging mammals. Furthermore, our initial data suggested that BBB breakdown may be one of the earliest biological indicators of brain aging, and as such elevated TGFβ signaling is apparently chronically active during the prolonged period of cognitive decline that encompasses the end of the lifespan – potentially over a period of decades in aging humans[55,59]. In such a context, in which the brain may undergo progressive and irreversible cognitive decline, it is uncertain whether acute interventions against TGFβ signaling, after the onset of cognitive dysfunction, would provide any benefit (even if the TGFβ played a causal role). And so, our primary questions in the final phase of our study were whether TGFβ signaling is causal in the pathology of brain aging, and whether interventions could effectively restore cognitive capacity.

To address these, we implemented two mechanistically distinct interventions in aging mice. To address the specificity of the TGFβ signaling pathway, we used transgenic mice in which the TGFβR could be inducible knocked out of astrocytes. The benefits of the system are that the intervention is highly targeted, not only to the first protein signaling step of the TGFβ pathway and but also to the exact cell type that we hypothesized to play the primary role in responding to BBB breakdown and albumin extravasation. However, a caveat is that such transgenic systems are limited by the efficiency of genetic recombination, and thus we expect levels of TGFβR to be reduced but not completely eliminated. As such, use of this intervention in aged mice might only weakly attenuate TGFβ signaling and fail to reverse the downstream biological outcomes. To address therapeutic potential, our other experimental intervention was a small molecule drug, IPW, which inhibits the TGFβR kinase, thus preventing the receptor from activating the TGFβ signaling cascade. Unlike the genetic KO intervention, we expect this drug intervention to be more potent (due to the ability to potentially inhibit all TGFβR across all cell types), but also less specific due to potential promiscuity of the drug for off-target inhibition of

other kinases. Together, use of these two distinct interventions – one targeted and mechanistically narrow, the other broad and therapeutically translational – were intended to provide a comprehensive and unambiguous assessment of the role of TGFβ signaling in age-related neural dysfunction.

4.2 Results

Transgenic inhibition of astrocytic TGFβ signaling reverses pathological outcomes in aging mice

We performed targeted experimental interventions to determine if TGFβ signaling plays a causal role in age-related outcomes. We aged cohorts of aTGFβR2/KO mice to early (12-16 months) and late (17-24 months) stages of aging, and then induced TGFβR2 KO in astrocytes to test their role in age-related cognitive dysfunction. We tested aged transgenic mice for spontaneous alternation in the T-maze, a hippocampal working memory task[168] that is impaired in aging rodents[169]. The task is optimal for assessing aging rodents because it can be performed rapidly without extensive training, is sensitive to even mild impairments in hippocampal function[170,171], and yet it is not affected by motor and vision impairments that may be present in aging mice[168]. At both early and late aging time points, aTGFβR2 KO mice performed significantly better than heterozygous controls (Figure 4.1A-B). We observed that at the early aging time point, several control mice showed notably high individual performance scores, and the overall mean performance of control mice decreased from early to late aging – in line with an expected age-related decline. This suggests that during early aging, there may be significant individual heterogeneity, with some individuals developing early BBB dysfunction, TGFβ signaling, and cognitive impairment, while others retain intact BBB and healthy levels of TGFβ signaling. To assess this, we performed T-maze in an additional cohort of early aging (12-16 month old) aTGFβR2 KO and heterozygous controls and collected dissected hippocampi to assess individual molecular outcomes. Using Western blot, we then quantified the level of hippocampal pSmad2 in each individual and correlated this biomarker of TGFβ signaling to the T-maze cognitive performance outcomes. Across both heterozygous (fl/+) and homozygous (fl/fl) genotypes, pSmad2 levels were negatively correlated with T-maze performance (Figure 4.1C). Together, these results show that targeted inhibition of the TGFβ signaling pathway, via induced KO in astrocytes, is sufficient to reverse the outcomes of seizure vulnerability and cognitive impairment in hippocampal spatial working memory in old mice, and that cognitive outcomes in a heterogeneous "mildly impaired" early aging cohort are correlated with the individual levels of TGFβ signaling.

A novel small molecule inhibitor of TGFβ signaling reverses molecular and functional outcomes in aging mice

These findings demonstrate the role of astrocytic TGFβ signaling in the pathogenesis of age-related neurological vulnerability and suggest the potential of TGFβR as a therapeutic target. Considering these therapeutic implications, we next tested the efficacy of a novel small molecule TGFβR1 kinase inhibitor, IPW. IPW has a promising clinical profile – including ability to

cross the BBB and good stability following oral dosing, making it suitable for once-per-day dosing to achieve inhibition of TGFβR signaling[172].

We then tested IPW as an intervention against TGFβ signaling in mice aged to 2 years old, near the end of the mouse lifespan. Immunofluorescent analysis of hippocampal sections from aged mice revealed that 5 days of treatment with IPW (20 mg/kg i.p.) reduced the number of astrocytes co-labeled with pSmad2 (Figure 4.2A). Western blot showed that old mice had significantly elevated levels of pSmad2, and 5 days of IPW treatment in aged mice reduced pSmad2 back to the baseline level seen in healthy, young mice (Figure 4.2B). Furthermore, IPW treatment reduced the downstream outputs associated with astrocytic TGFβ signaling, including levels of TGFβ1 and the marker of astrocyte activation, GFAP (Figure 4.2C-D). To examine outcomes related to network dysfunction, we performed a longitudinal study on the young and old mice that had been segmented by GMM clustering into l-SWE and h-SWE subgroups, to assess their individual trajectories following treatment. After 5 days of baseline recording, mice were treated for 5 days with IPW, followed by 5 days of "washout" after halting IPW treatment (Figure 4.2E). IPW treatment had no effect on the l-SWE old or on young mice, which continued to show normal ECoG activity. In contrast, IPW treatment ameliorated aberrant ECoG activity in the h-SWE group, reducing the number of SWEs to a level seen in young mice. This effect of IPW persisted throughout the 5-day washout period. A separate cohort of old mice, classified with h-SWE ECoG activity, underwent the same experiment, except with administration of vehicle control; vehicle injection had no effect on aberrant slow-wave activity (Figure 4.2F).

Given the effects of IPW on inhibiting TGFβ signaling and reversing electrophysiological markers of neural network dysfunction, we next assessed functional outcomes in naturally aged mice. Following 7 days of IPW treatment, aged mice also showed significant improvement in the spontaneous alternation T-maze task (Figure 4.2G), again consistent with the findings from genetic aTGFβR KO mice. To further assess cognitive outcomes, we performed the novel object recognition task, which is also sensitive to age-related memory decline, but is less dependent on hippocampal function than spatial maze tasks[173,174]. After 7 days of IPW treatment, aged mice showed significant improvement in object memory versus vehicle-treated controls (Figure 4.2H).

These results provide evidence for a mechanism that directly links brain microvascular pathology and BBB dysfunction, which occur widely in mammalian aging, to age-related neural and cognitive dysfunction. We showed that astrocytic TGFβ signaling functions as a regulatory cascade that induces changes in molecular, electrophysiological, and behavioral outcomes. Key findings are that 1) BBB dysfunction appears as early as middle age, with a time course that is well-matched to first appearance of mild age-related symptoms and precedes late-stage disease; 2) induction of TGFβ signaling with albumin in young mice is sufficient to cause the neurological outcomes observed in old mice; and 3) intervention against TGFβ signaling, either by targeted genetic KO in astrocytes or by small molecule drug, are sufficient to reverse neurological markers and functional outcomes in aging.

4.3 Discussion

We inhibited TGFβ signaling in aged mice using two distinct interventions, and found that both approaches achieved similar efficacy in restoring cognitive ability, indicating that the TGFβ signaling pathway not only plays a major role in age-related dysfunction, but also that it holds therapeutic potential. Notably, the small molecule drug intervention restored a "youthful" profile in biomarkers spanning levels of analysis – from the level of TGFβ signaling itself (pSmad2 protein), to the ECoG recordings of brain activity, to cognitive behavior tasks. We also observed notable individual variability in the outcomes of aging mice, with some mice remaining apparently healthy even in old age when profiled by ECoG or cognitive behavioral assessment. Such heterogeneity is also present in aging humans, and our results raise the possibility that vascular leakiness is a trigger affecting individual outcomes, given our finding that individual cognitive outcomes in mice inversely correlate with the severity of TGFβ signaling (pSmad2). In humans, the severity of BBB breakdown can be quantified by contrast-enhanced MRI[59,175,176] and by analysis of blood proteins in the CSF[55]. Thus a key extension of this research will be future studies aimed at determining whether BBB status is predictive of individual cognitive trajectories in aging humans.

4.4 Methods

Molecular biology assays. Immunofluorescent staining, microscopy, and Western blot were performed as described in chapter 3 methods.

ECoG. ECoG was performed as described in chapter 3 methods. Mice were recorded for 15 days, starting 1 week after surgery, and administered IPW (20 mg/kg) on days 6-10 of recording. To detect SWE, ECoG signals were buffered into 2 sec long epochs with an overlap of 1 sec. Fast Fourier Transform (FFT) was applied and the frequency of median power was extracted for each epoch. Thus, an event was considered as a SWE if its frequency of median power was less than 5 Hz for 10 consecutive seconds or more. FFT was also applied for the entire recording period to analyze relative power across the frequency spectrum of 1-20 Hz.

Animal care and transgenic mice. All animal procedures were approved by the institutional animal care committees. Animals were housed with a 12:12 light:dark cycle with food and water available ad libitum. Strains of transgenic mice are described in chapter 3 methods.

Behavior assessments. Spatial working memory was tested by spontaneous alternation in a T-maze constructed of black plastic, with stem (50 x 16 cm) and two arms (50 x 10 cm). A vertical divider was placed at the midline of the stem exit, thus creating two entryways leading to either the right or left arms. Naïve mice were placed at the beginning of the stem and allowed to freely roam until opting to enter either the right or left arm. Upon crossing the threshold of the chosen arm, a door was lowered, and the mouse was contained to the chosen arm for 30 sec. Then, the mouse was returned to the stem and the door raised, allowing the next choice trial to commence immediately. After 10 sequential trials, the mouse was returned to the home cage. "Correct" alternation choices were scored when the mouse chose the arm opposite of that chosen in the previous trial, and percent correct was calculated as (total correct choices) / (total number of completed trials). In the event that a mouse did not leave the stem within 60 sec, it was removed from the apparatus for 30 sec, and then reset in the stem to start a new trial. These "incomplete" trials were not counted in scoring. Object memory

was tested in the novel object task, using a 3-day protocol consisting of a 10 min trial on each day, recorded by overhead video. On day 1 mice were habituated to a square testing chamber, constructed of white plastic (50 x 50 cm), with two unfamiliar objects placed inside. On day 2, the previous objects were removed and 3 new objects were placed in a "L" configuration, equidistant from each other and the walls. On day 3, one of the objects was removed and replaced by a new object, thus leaving 2 familiar objects and 1 novel object. A blind observer quantified duration of time spent investigating each object (scoring criteria were mouse nose oriented towards object at a distance of 1 cm or less), and percentage of time at the novel object was quantified as (duration investigating novel object) / (total duration investigating all three objects). The set objects were chosen from common items such as lab bottles, pipette boxes, cups (placed open-side down), etc., that were of similar dimensions but varied in shape, color, and material. The sequence and position of objects across trials was identical for all mice.

Statistics and data. Data collection and preparation was performed by researchers who were blind to experimental conditions, and the lead and corresponding authors were responsible for experiment design, conducting statistical analysis, and unblinding the final results. All graphs are plotted showing mean and SE. Two sample comparisons were performed by student's t-test or Mann-Whitney test, and multiple group comparisons were conducted by ANOVA or Kruskal–Wallis test, followed by post-hoc testing to compare individual groups when a main effect was detected. Multiple correction comparisons were used as described in figure legends. Seizure progression in the PTZ experiments was analyzed by two-way ANOVA, and linear regression was used to calculate regression slopes. Differences in regression slope were also compared by ANOVA. Different subgroups that were observed in ECoG recordings were first classified by an unbiased Gaussian mixed model, and then inferential statistics were performed on the subsequent groups. For all inferential statistics, two-tailed tests were used and significance thresholds were set at $p<0.05$.

4.5 Figures

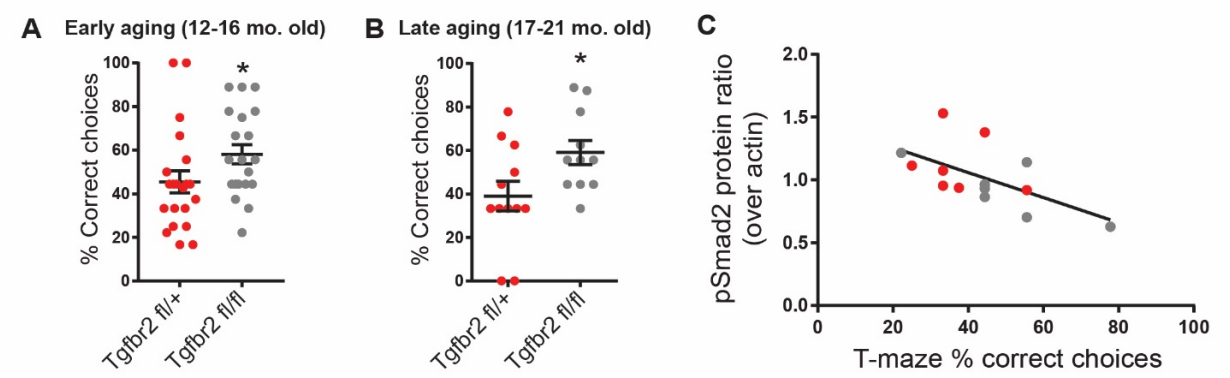

Figure 4.1. Knock-out of astrocytic TGFβ reverses cognitive outcomes in aging mice. KO was induced in early (12-16 mo.) and late (17-24 mo.) aged mice, and T-maze testing was performed 35 days later. (K-L) In T-maze, aTGFβR KO mice showed significantly better working memory performance compared to heterozygous controls at in both early aging (12-16 months old, Mann-Whitney test, $p = 0.0273$, $n = 21$ $Tgfbr2^{fl/+}$, 20 $Tgfbr2^{fl/fl}$) and late aging assessments (t-test, $p = 0.035$ $n = 12$ $Tgfbr2^{fl/+}$, 11 $Tgfbr2^{fl/fl}$). (M) An additional cohort of 12-16 month old mice was tested in T-maze, and hippocampi were dissected for Western blot analysis of TGFβ signaling (pSmad2) to assess individual outcomes. T-maze performance was negatively correlated with levels of pSmad2 signaling across individuals of both genotypes ($Tgfbr2^{fl/+}$ and $Tgfbr2^{fl/fl}$) (Pearson's correlation, $r^2 = 0.3577$, $p = 0.024$, $n = 14$).

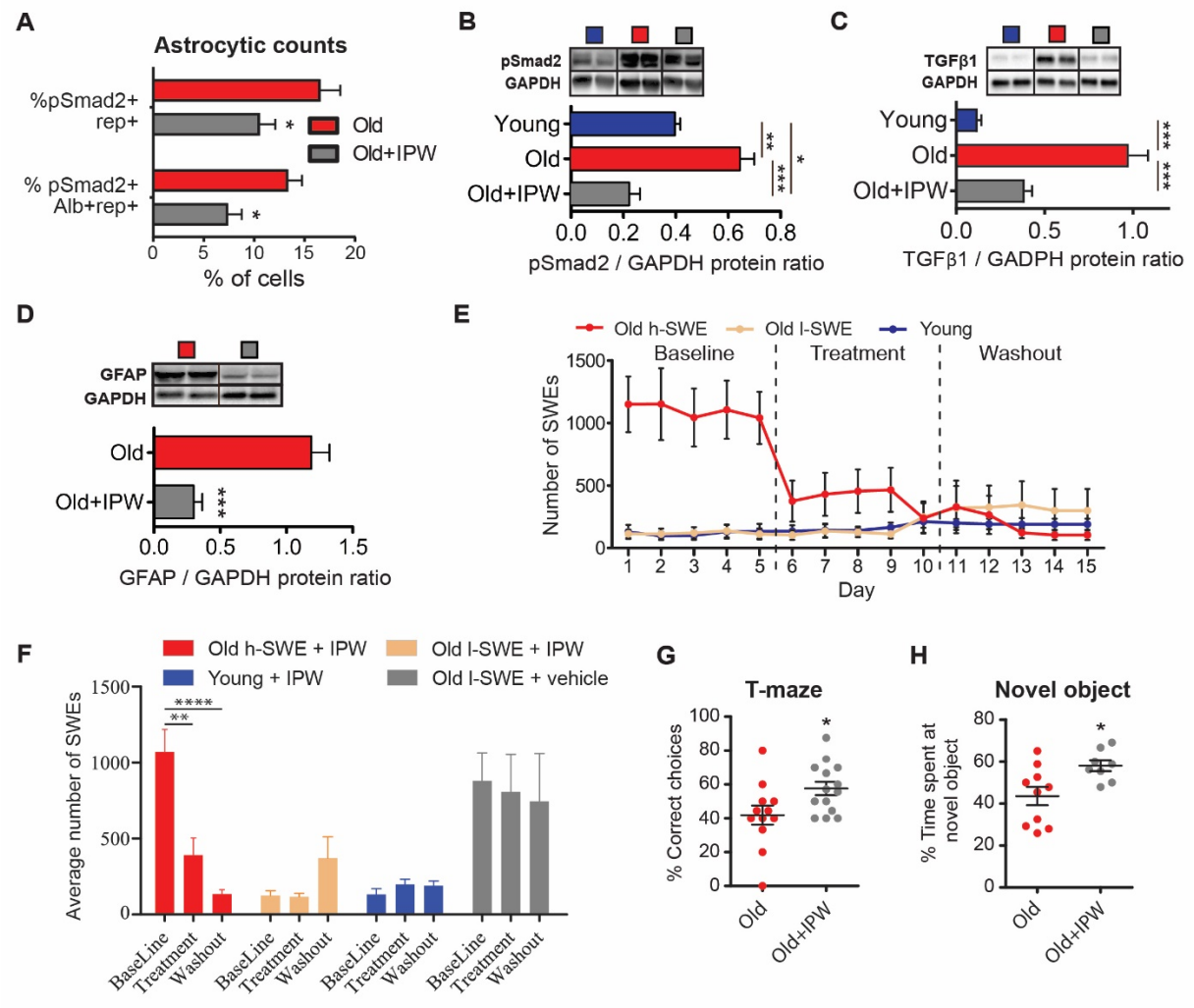

Figure 4.2. IPW reverses TGFβ signaling, aberrant neural activity, and cognitive impairment in aged mice. (A) Aged Rep-Aldh1L1 mice were treated 7 days of IPW (20 mg/kg, i.p.) or vehicle, followed by immunofluorescent staining for pSmad2 and albumin (Alb). IPW significantly reduced fraction of astrocytes (rep+) that co-labeled with pSmad2, and the fraction of cells triple labeled with pSmad2, Alb, and rep (t-test with Holm-Bonferroni correction, n = 5 old, 6 old+IPW). (B) Densitometry of Western blot shows that IPW restored levels of elevated pSmad2 protein in old mice, to the level of young mice (ANOVA, main effect $p<0.0001$, with Tukey's posttest, n = 4). IPW treatment also reduced levels of (C) TGFβ1 in aged mice (ANOVA, main effect $p<0.0001$, with Tukey's posttest, n = 4) and (D) GFAP protein, a marker of activated astrocytes (t-test, $p = 0.0004$, n = 4 old, 5 old+IPW). (E) Continuous ECoG recording over 15 days was used to investigate effects of IPW on aberrant network activity. During 5 days of baseline recording, clustering analysis was used to classify old mice as affected by high numbers of SWEs (h-SWE, n = 6), or low numbers of SWEs (l-SWE, n = 6), similar to the level of young mice (n = 5). In the subsequent 5 days of recording, IPW treatment restored a youthful ECoG profile in the affected mice, reducing SWEs (Dunn's multiple comparisons test $p = 0.0098$) to the level of young mice. After IPW dosing was halted, the old mice retained a l-SWE ECoG profile throughout the "washout" period (Dunn's multiple comparisons test baseline vs washout $p = 0.0002$). (F) Average number of SWEs for all groups during the final two days of each treatment period (Baseline prior to injection, treatment with daily injection of IPW or vehicle control, and washout with cessation of injections). Treatment significantly reduces SWEs in the h-SWE group, whereas no changes in SWEs were observed in any other group. (G) 7 days of IPW treatment improved cognitive scores of old mice in the T-maze (t-test, $p = 0.028$, n = 12 vehicle, 14 IPW) and (H) novel object memory tasks (t-test, $p = 0.017$, n = 10 vehicle, 8 IPW).

Chapter 5. Discussion

Adapted from: Friedman AR, Cacheaux L, Ivens S, Kaufer D, 2011. Complex interactions between stress and epileptogenic pathways. *Cardiovasc Psychiatry Neurol*, 2011:461263; from: Chetty S*, Friedman AR*, Taravosh-Lahn K, Kirby ED, Mirescu C, Guo F, Krupik D, Nicholas A, Geraghty AC, Krishnamurthy A, Tsai MK, Covarrubias D, Wong AT, Francis DD, Sapolsky RM, Palmer TD, Pleasure D, and Kaufer D, 2014. Stress and glucocorticoids promote oligodendrogenesis in the adult hippocampus. *Mol Psy*, 19(12):1275-83; and from: Senatorov VV Jr*, Friedman AR*, Milikovsky DZ, Ofer J, Saar-Ashkenazy R, Charbash A, Jahan N, Chin G, Mihaly E, Lin JM, Ramsay HJ, Moghbel A, Preininger MK, Eddings CR, Harrison HV, Veksler R, Becker A, Hart B, Rogawski MA, Dillin A, Friedman A, and Kaufer D, 2018. Blood-brain barrier breakdown during aging causes chronic but reversible neural dysfunction via TGF-beta signaling. Manuscript in preparation. *Equal contribution.

One of my fundamental scientific interests, which has been the focus of my graduate work and will remain central in my future career, is to understand how environmental triggers and lifetime experiences are embedded in the brain to cause later disease. In particular, much of current neuroscience in disease models is focused on the late-stage symptoms that we associate with the given disease – for example mental illness research focused on drugs that modulate mood, epilepsy research focused on suppressing and controlling seizures, or dementia research focused on late-stage degenerative markers and approaches for boosting neural function. My goal is to instead understand the early biological mechanisms that precede disease onset, to seek new approaches for preventing disease before it starts by modifying the underlying disease progression. My work is particularly relevant to human health and supports a parallel goal of performing translational research. I believe that we can transform our healthcare system by a shift towards biologically designed preventative treatments and away from chronically prescribed drugs aimed at managing disease symptoms without modifying underlying disease etiology, and I want to play a role in the fundamental and translational academic research that drives this shift. In particular, I believe it is imperative that academics are able to work on the interface of translational research and move their findings out of the lab so that they can actually reach the clinic and change patient outcomes.

By taking a “long view,” I have tried to understand how brains change over time, and in particular how pathological changes can slowly accumulate to push the brain out of a healthy state and towards onset of neurological disease. One surprising insight from this perspective is that the way in which the brain responds to a wide variety of insults and injuries, from stress to physical damage to aging, may be somewhat stereotypical, and hence a wide range of neurological diseases that are thought to be distinct may actually have shared underlying risk factors and biological antecedents. If so, then new disease-modifying therapeutic approaches that target these underlying mechanisms may not only be more effective than current, symptom-modifying drugs, but also more broadly applicable and potentially useful in many types of neurological disease. Here, I discuss how some of the key biological mechanisms characterized in my graduate work may be relevant toward understanding, and ultimately treating, human neurological diseases.

The impact of structural white matter changes in the brain

Our work showed that stress hormone acts as a signal that impacts production of oligodendrocytes in the brain. As the cells that are responsible for myelinating neural axons, oligodendrocytes potentially play a major role in regulating the strength of integrity of neural signaling. Our work followed the effects of stress on oligodendrogenesis over relatively short timespans (up to one month), but this in turn raises the larger question: what are the potential implications of stressful incidents in sculpting brain composition over the entire lifespan? In particular, early life stress may also have a profound impact on the development of white matter in the brain. Work in our lab and others indicates that stress may increase or decrease myelination, depending on developmental stage and other unknown factors[177]. These paradoxical findings are echoed by literature showing that GCs induce *in vitro* oligodendrocyte precursor cells (OPCs) to differentiate into mature oligodendrocytes[27,178,179], and promote oligodendrocyte survival[28], yet total removal of GCs by adrenalectomy results in hypermyelination[180] while prenatal GC treatment delays myelination in sheep[181]. Early life stress is one of the strongest risk factors for the later development of mental illness in adulthood[21], but these same mental illnesses are also associated with altered patterns of myelination in the brain[38–42]. If early life stress does result in altered myelination, it would constitute a startling and underappreciated similarity not only to myelin changes associated with mental illness, but also to a variety of other neurological diseases. Delayed myelination is a hallmark of infantile spasms[182] and other seizure disorders[183], and several genetic hypomyelination disorders or manipulations include severe seizure symptoms[184,185]. In these models, treatment is associated with white matter recovery: amino acid supplement of patients with a serine biosynthesis disorder resulted in restoration of white matter and major seizure reduction[186,187]. Pharmacologically (L-allylglycine, bicuculline, and kainic acid) or electrically induced seizures also cause demyelination[188,189], and alterations of white matter have been associated with both symptomatic and idiopathic epilepsy[190–192] and with hippocampal sclerosis[193]. Indeed, while glia have received a surge of recent interest for causal roles in epilepsy, this attention has focused almost exclusively on astrocytes. Possible roles for oligodendrocytes remain largely uninvestigated. Similarly, changes in myelination are significantly associated with cognitive decline in aging individuals[124,125]. Together, these observations suggest that stress-induced changes in myelination could potentially contribute to fundamental neural impairments that in turn could create vulnerabilities for emergence of multiple, disparate symptoms and diseases.

Vascular permeability as a trigger for neurological disease and a potential treatment strategy

Our experiments uncovered a foundational mechanism linking BBB decline to neural dysfunction in aging, via TGFβ signaling which acts as a potent signal to suppress neural function. This same mechanism, including BBB breakdown and TGFβ signaling, appears to be active in many other disease contexts including head injury, stroke, and neurdegenerative disease such as Alzheimer's disease, amytrophic later sclerosis and multiple sclerosis[52,56–58,61,62,84,194–196], which all have remarkably similar secondary outcomes including cognitive impairment and risk for epilepsy. But unlike acute injury, our finding of chronic BBB leakiness in

aging raises several critical questions: what causes BBB decline itself? And is BBB decline causal to, concurrent with, or an outcome of (or independent from) other well-known mechanisms of aging such as inflammation, reactive oxidation stress and metabolic failures, proteasome senescence, DNA degradation, etc.[197–202]? Our work, while novel in focusing on microvascular integrity and interactions within the neurovascular unit, can be placed in context of some of these mechanisms. For example, activation of astrocytes and gliosis has been shown to be a key step in many different aging diseases, with astrocytes playing potent roles in controlling neuroinflammation, neural functions including synaptic plasticity, senescence, and neurodegeneration[203–207]; we show that BBB dysfunction may be an early step causing or contributing to activation of astrocytes and the ensuing inflammatory response. Similarly, several previous studies have reported increased TGFβ signaling in aging[73–76], and suggested that it could be a primary regulatory factor inducing aged neural phenotypes; we show that astrocytic uptake of albumin may be one of the earliest steps first inducing this age-related TGFβ cascade. But beyond such mechanistic rodent studies, only large-scale human epidemiological studies will provide the necessary and critical insight into the relationship between BBB status, other known biomarkers, and disease outcomes. We argue that such translation of this research to the human context holds exciting potential for age-related diseases, like dementia and epilepsy, that continue to be poorly understood and go without effective treatment.

Could inhibition of TGFβ signaling, as a strategy to counteract the detrimental consequences of age-related BBB dysfunction, hold therapeutic potential? One of the major challenges (and causes of failure) in treating progressive neurological diseases is that patients decline over time, and thus may accumulate irreversible damage by the time of diagnosis. Considering that BBB dysfunction begins relatively early in aging, if it were necessary to chronically treat patients with TGFβ inhibitors to prevent future damage, that would present a considerable clinical challenge. However, we found that one week of treatment with a TGFβR inhibitor fully reversed the pathological outcomes that we had observed in aging mice, including elevated TGFβ signaling, aberrant ECoG activity, and cognitive dysfunction. This suggests that the aging brain may retain considerable capacity for plasticity and function, which may be chronically suppressed (but not irreversibly lost) by BBB leakiness and its inflammatory fallout.

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