UCSF
UC San Francisco Electronic Theses and Dissertations

Title
Systemic regulation of synaptic, regenerative, and cognitive dysfunction during aging

Permalink
https://escholarship.org/uc/item/00t9b6cz

Author
Smith, Lucas Kenneth

Publication Date
2020

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Systemic regulation of synaptic, regenerative, and cognitive dysfunction during aging

by
Lucas Smith

DISSERTATION
Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

DocuSigned by:
Susanna Rosi
211375A844174DF...

Susanna Rosi
Chair

DocuSigned by:
Dena Dubal
[illegible]

Dena Dubal

DocuSigned by:
A. Brack
[illegible]

Andrew Brack

DocuSigned by:
Saul Villeda
711626DA5837464...

Saul Villeda

Committee Members

Copyright 2020

by

Lucas K. Smith

Acknowledgements

Parts of the introductory chapter are modified from Smith LK, White CW III, and Villeda SA. (2018). *Cell and Tissue Research*. 371(1): 105–113. doi: 10.1007/s00441-017-2715-8. Part of **Figure 1.1** has been adapted from Fan X, Wheatley EG, and Villeda SA. (2017). *Annual Reviews Neurosciences*. 40: 251-272. doi: 10.1146/annurev-neuro-072116-031357. Chapter 2 is adapted from Smith LK, Verovskaya E, Bieri G, Horowitz AM, Ungern-Sternberg SNI, Lin K, Seizer P, Passegue E, Villeda SA. (2020). *Aging Cell*. doi:10.1111/acel.13192

Many people have contributed to this dissertation and to my scientific training. I would like to first thank my thesis advisor, Saul Villeda, for his support, inspiration, and expertise. Saul has created a laboratory environment containing unlimited opportunity. I thank Saul for the well-rounded scientific training, the freedom to pursue interesting questions, and for his guidance.

I have been fortunate to benefit from many amazing mentors over the years. These include the members of my thesis committee, Susanna Rosi, Dena Dubal, and Andrew Brack, who have provided critical scientific insights and advice towards advancing my studies. I am extremely grateful to each of you. Additionally, I am thankful of my first PI, Jerome Deval, who hired me out of college with no experience. The excellent training I received from Jerome was instrumental in sparking my scientific journey, and I would not have pursued a career in science without it.

I would also like to thank the Villeda lab, who have created an excellent and stimulating work environment. The members of the Villeda lab are incredibly open, collaborative, and generous. I would like to particularly thank, Alana Horowitz, Gregor Bieri, Karin Lin, and Shelly Fan who all directly helped with the studies presented in this dissertation, but I am grateful to every member of the lab. You each helped advance this research and enrich my life for the past years.

This dissertation would not have been possible without collaboration. In particular, I would like to thank Emmanuelle Passegué who provided critical scientific expertise and who helped hone the inception of this work, as the chair of my qualifying exam committee. I would also like to thank the Passegué lab for training in HSC biology and transplantation techniques. In particular, I thank Evgenia Verovskaya for being incredibly helpful and generous, and for doing HSC transplantation experiments with me until 5am in the morning.

I would like to thank my friends in the BMS program and at UCSF at large, who have taught me so much. I feel fortunate to have such an incredible group of colleagues who inspire me with their genius, work ethic, and consideration. In particular, I thank Elze, Adair, and Eric for late night chicken dinners in the foggy Outer Sunset, and endless scientific discussion.

Last, and most importantly, I would like to thank my family. I thank my mom and dad, Linda and Steve, for a lifetime of unconditional love. I thank my brothers Connor and Owen for paving the way and for unwavering support. I also thank my partner, Mei-Lani and the bad-dog-Barbie who help to make life good.

Systemic regulation of synaptic, regenerative, and cognitive dysfunction during aging

Lucas K. Smith

Abstract

Aging is a prominent risk factor for cognitive decline and neurodegenerative disease. Particularly sensitive to the effects of aging are functions governed by the hippocampus and indeed, impairments in hippocampal-dependent episodic and spatial memories have been observed in aged rodents, monkeys, and humans. While the mechanisms by which age-related cognitive impairments emerge are multifaceted, neuronal dysfunction and impaired plasticity are frequent hallmarks of brain aging thought to underlie cognitive decline. In the hippocampus, this is manifested as a loss of neurogenesis and decreased long-term potentiation, which is accompanied by corresponding decreases in synaptic density and synaptic protein expression. As the human population ages, understanding mechanisms governing hippocampal aging is increasingly important as means of identifying therapeutic targets to slow or even reverse aging in the brain. While significant work has characterized intrinsic cellular changes that contribute to age-related loss of neurogenesis, synaptic dysfunction, and cognitive decline, recent studies posit age-related changes in blood as a critical regulator of brain aging. Indeed, emerging studies utilizing the model of heterochronic parabiosis - in which the circulatory system of a young and old mouse is surgically joined - have revealed bidirectional pro- and anti- aging effects of old or young blood exposure on hippocampal neurogenesis, synaptic plasticity, and cognition. To date, studies have focused on age-related changes in blood-borne factors as regulators of hippocampal aging, and whether other components of blood contribute to hippocampal aging remains to be fully elucidated. Here, we assess the role of cellular aging of the hematopoietic system in driving hippocampal aging (Chapter 2) and test whether circulating

exosomes in young blood contain rejuvenating capacity on the aged brain (Chapter 3). We find that cellular aging of the hematopoietic system, and downstream changes in blood-borne factors, drive age-related impairments in hippocampal cognition. Additionally we find that systemic administration of young plasma-derived exosomes fail to rejuvenate the aged hippocampus, but increase synaptic protein expression in the aged cortex.

Table of contents

Chapter 1. The systemic environment regulates hippocampal aging…… 1

1.1 The mammalian hippocampus and aging…… 1

1.2 Old blood promotes hippocampal aging…… 3

1.3 Young blood rejuvenates the aged hippocampus…… 5

1.4 Aims of this study…….. 7

1.5 References…… 10

Chapter 2. The aged hematopoietic system promotes hippocampal-dependent cognitive decline…… 18

2.1 Author contributions…….. 19

2.2 Abstract…….. 20

2.3 Introduction…… 21

2.4 Results…… 21

2.5 Discussion…….. 26

2.6 References…… 34

2.7 Materials and Methods…… 37

Chapter 3. Young plasma and young plasma-derived exosomes differentially affect brain aging in a regionally specific manner…… 47

3.1 Author contributions…….. 48

3.2 Abstract……... 49

3.3 Introduction…… 50

3.4 Results…… 51

3.5 Discussion…….. 55

3.6 References…… 62

3.7 Materials and methods…… 66

List of Figures

Chapter 1

Figure 1.1 Age-related changes in blood regulate hippocampal neurogenesis, neuronal function, and memory. …….. 9

Chapter 2

Figure 2.1 Establishment of a heterochronic HSC reconstitution model…….. 27

Figure 2.2 The aged hematopoietic system promotes hippocampal-dependent cognitive decline…… 28

Figure 2.3 The aged hematopoietic system promotes cellular hallmarks of hippocampal aging…….. 29

Figure 2.4 Low infiltration of peripheral immune cells in the dentate gyrus of isochronic and heterochronic HSC reconstituted mice. …… 30

Figure 2.5 Identification of CyPA as a candidate pro-aging factor elevated in the blood of heterchronic HSC reconstituted young mice. ……..……..……..…….. 31

Figure 2.6 Systemic overexpression of CyPA promotes cellular and cognitive hallmarks of hippocampal aging in young mice. ……..……..……..……..……. 32

Figure 2.7 Inhibition of systemic CyPA ameliorates cellular and cognitive hallmarks of hippocampal aging..……..……..……..……..…… 33

Chapter 3

Figure 3.1 Young plasma administration rejuvenates the aged hippocampus. ……... 56

Figure 3.2 Isolation of exosomes from young mouse plasma. ……..……. 57

Figure 3.3 Young plasma-derived exosomes do not rejuvenate the aged hippocampus…… 58

Figure 3.4 Young plasma administration increases expression of key proteins involved in synaptic function in the cortex of aged mice. ……..……..……..……... 60

Figure 3.5 Young plasma-derived exosomes increase synaptic marker expression in the cortex of aged mice. ……..……..……..……..……..……..…….. 61

List of Abbreviations

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

B2m – Beta-2-microglobulin

BDNF – Brain derived neurotrophic factor

BrdU – Bromo Deoxyuridine

CA – Cornu Ammonis

CCL11 – C-C motif chemokine ligand 11

CREB – cAMP response element-binding protein

CyPA – Cyclophilin A

DCX – Doublecourtin

DG – Dentate Gyrus

EC – Entorhinal cortex

FC – Fear conditioning

GFP – Green fluorescent protein

HDTVI – Hydrodynamic tail vein injection

Het – Heterochronic

HSC – Hematopoietic stem cell

Iso – Isochronic

LTP – Long-term potentiation

NeuN – Neuronal Nuclear antigen

NMDA – *N*-methyl-d-aspartate

NPC – Neural progenitor cell

NSC – Neural stem cell

OCN – Osteocalcin

PBS – Phosphate buffered saline

PSD95 – Postsynaptic density protein 95

RAWM – Radial arm water maze

SASP – Senescence-associated secretory phenotype

SGZ – Subgranular zone

TGF-B - Transforming growth factor beta

TIMP2 - Tissue inhibitor of metalloproteinases 2

VCAM-1 - Vascular cell adhesion molecule 1

The systemic environment regulates hippocampal aging

Lucas K. Smith

1.1 The mammalian hippocampus and aging

Mammalian aging is associated with the emergence of diverse and widespread impairments in tissue homeostasis and regenerative capacity[1,2]. In the brain, aging leads to a decline in cognitive processes[3] and is the prominent risk factor for dementia-related neurodegenerative disease[4]. One region of the brain that is particularly susceptible to the effects of aging is the hippocampus, which governs important cognitive functions including spatial navigation and episodic memory[5]. Sensory information is processed through the hippocampus primarily by glutamatergic signaling at dendritic spines along the trisynaptic circuit[6]. Specifically, inputs from the entorhinal cortex (EC) connect to granule neurons in the dentate gyrus (DG), which, in turn, project to pyramidal neurons in the Cornu Ammonis region 3 (CA3), via mossy fiber axons. CA3 neurons project to pyramidal neurons in the CA1 by Schaffer collateral projections, and CA1 outputs to the EC. In response to stimulation, neurons undergo structural and functional changes that promotes synaptic strengthening, a process termed long-term potentiation (LTP). This process is thought to be a cellular underpinning of hippocampal learning and memory[7], and is dependent on changes in the glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and *N*-methyl-d-aspartate (NMDA) receptor[8,9].

The mammalian hippocampus is a unique brain region, in that it retains neurogenic capacity throughout life[10]. The process of neurogenesis begins when neural stem cells (NSCs), residing in the subgranular zone (SGZ) of the DG, enter a proliferative neuroblast state and give rise to immature neurons that migrate into the granule cell layer of the DG. There, newly formed neurons extend processes and ultimately integrate into the hippocampal circuit projecting to the

CA3. While a recent histological study has challenged the existence of neurogenesis in the adult human brain[11], many other studies employing 14C or BrdU/IdU birthdating[12–14], and histological approaches[15–17] support the existence of neurogenesis as a robust process in the adult human hippocampus. Indeed, studies have estimated that the adult human hippocampus contains thousands of immature neurons at various stages of maturation[16] and produces a median of 700 new neurons per day[12].

Adult neurogenesis has been implicated in cognitive function, and recent rodent studies employing increasingly refined approaches have strengthened this link. For example, impaired spatial memory has been reported in mice after genetic and pharmacological ablation of proliferating neural progenitor cells (NPCs), or after optogenetic silencing of newborn granule cell neurons[18,19]. Moreover, lentiviral mediated enhancement of neurogenesis has been shown to improve contextual learning and memory in aged mice[20]. Interestingly, recent histological studies suggest that hippocampal neurogenesis may be similarly relevant to cognition in humans. For example, levels of hippocampal neurogenesis have recently been shown to correlate with cognition in aged humans[17]. Additionally, decreased hippocampal neurogenesis has been observed in Alzheimer's disease patients compared to cognitively normal age-matched controls, with the levels of neurogenesis showing an inverse correlation with disease severity[16].

Aging is associated with the emergence of impairments in neuronal and neurogenic function in the hippocampus, accompanied by concomitant cognitive decline and increased risk for neurodegenerative disease[21]. At the neuronal level, aging is associated with impaired LTP, which has been demonstrated by electrophysiological recordings in hippocampal slices[22,23]. Potentially contributing to the age-related decline in LTP, decreased expression of NMDA and AMPA receptor subunits has been reported in the aged hippocampus[24–26]. At a structural level, decreased dendritic spine density has been frequently observed in the hippocampus of aged

rodents and primates[27], and is associated with age-related cognitive impairments[28]. Additionally, aging is associated with a marked decrease in hippocampal neurogenesis. This decline has been well characterized in rodents[29,30], and has been observed in a number of human studies using histological and birthdating methods[12,16,31]. Notably, aging results in a decline in the entire neurogenic cascade, and is associated with a decrease in the number of NSCs, neuroblasts, and newly born neurons in the DG of mice and humans[12,16,29,31]. Moreover, recent work has found that aged hippocampal NSCs are more quiescent than young NSCs[32], suggesting that the age-related decrease in hippocampal neurogenesis reflects both a decline in NSC number and function.

Together, these cellular changes are thought to contribute to the emergence of cognitive impairments in aging[33]. Behavioral testing in animal models has revealed age-related impairments in hippocampal-dependent spatial memory and contextual fear memory[34,35]. Similarly, in healthy humans, hippocampal-dependent spatial and episodic learning and memory decrease during aging[36–38].

As the human population is ages[39], identifying the drivers of hippocampal aging is increasingly important for the therapeutic extension of healthspan. To that end, recent studies have identified the systemic environment as an important regulator of neuronal function, neurogenesis, and cognition in the aged hippocampus (**Figure 1.1**). Studies using the model of heterochronic parabiosis, in which the circulatory systems of a young and aged mouse are surgically joined, have revealed bidirectional pro- and anti-aging effects of old and young blood exposure on the central nervous system, respectively[34,40–45]. Identifying the mechanisms underlying these effects is now of paramount importance.

1.2 Old blood promotes hippocampal aging

Exposure to old blood in the model of heterochronic parabiosis has been shown to promote a number of cellular hallmarks of aging in the young hippocampus. At the neuronal

level, old blood impairs LTP in the young brain, as assessed by electrophysiological recordings on hippocampal slices[34]. Moreover, old blood exposure inhibits hippocampal neurogenesis, leading to a decrease in the numbers of NPCs, immature neurons, and newly formed mature neurons in the DG of young mice[34,45]. Corroborating these findings, in an independent, catheter-based model of heterochronic blood exchange, short-term exposure to old blood has been shown to exert anti-neurogenic effects on the young hippocampus[46]. In addition to the pro-aging effects of old blood exposure observed in heterochronic parabiosis and catheter-based blood exchange studies, systemic administration of old blood plasma has been shown to inhibit hippocampal neurogenesis in young mice and to impair hippocampal-dependent cognition in the radial arm water maze (RAWM) and contextual fear conditioning (FC) behavioral paradigms[34,44]. These studies posit age-related changes in soluble blood-borne factors as a mediator of the pro-aging effect of old blood.

Initial studies have identified a number of candidate pro-aging factors elevated in the blood of aged mice and young heterochronic parabionts, of which, Beta-2-microglobulin (B2M) and C-C motif chemokine ligand 11 (CCL11) are additionally elevated in the blood of aging human subjects[34,45,47–49]. Interestingly, systemic administration of B2M or CCL11 in young mice promotes age-related impairments in hippocampal neurogenesis and cognition[34,45]. Moreover, systemic administration of CCL11 has been shown to drive an age-related decrease in dendritic spine density in the hippocampus of young mice[50]. Recent studies have identified additional pro-aging immune factors, including vascular cell adhesion molecule 1 (VCAM-1) and transforming growth factor beta (TGF-B), which are elevated in the blood of aged mice[44,51]. Systemic inhibition of VCAM-1 or TGF-B in aged mice, via an antibody or pharmacological mediated approach, respectively, has been shown to increase neurogenesis and/or improve hippocampal cognition[44,51]. Collectively, these studies posit the accumulation of pro-aging factors in blood as a driver of hippocampal aging, and demonstrate the therapeutic potential of inhibiting such factors.

Notably, all blood-borne pro-aging factors identified to date are immune related molecules, positing age-related changes in the peripheral immune system as a driver of hippocampal aging[52]. In blood, aging drives many functional and structural changes that result in impaired immunity, increased rates of anemia, and increased incidence of myeloid malignancies[53]. Specifically, aging results in decreased production of naïve T cells, and a peripheral expansion of senescent memory and effector T cells[54] that may promote an inflammatory systemic milieu as a result of their elevated cytokine production[55–57]. Similarly, there is reduced production of naïve B cells, and an accumulation of memory B cells with increased production of autoreactive antibodies[54]. Accompanying these changes to the adaptive immune system, is the progressive dysregulation of the innate immune system, which includes the functional decline of neutrophils, natural killer cells, monocytes, and dendritic cells[58]. Together, these changes are poised to contribute to the accumulation of pro-aging factors in old blood. Indeed, increased transcription the pro-aging factor, B2M has been reported in aged human peripheral blood mononuclear cells (PBMCs)[59]. However, whether cellular aging in blood contributes to hippocampal aging remains to be elucidated.

1.3 Young blood rejuvenates the aged hippocampus

In addition to elucidating the pro-aging effects of old blood exposure on the young hippocampus, studies using the model of heterochronic parabiosis have revealed broad rejuvenating effects of young blood exposure on the aged brain[34,40,42,43]. Indeed, during parabiosis, young blood has been shown to increase LTP, dendritic spine density, and neurogenesis in the aged hippocampus[34,43]. Additionally, administration of young mouse plasma has been shown to improve hippocampal-dependent cognition in aged mice[43,60], suggesting that the benefits of young blood exposure are, at least in part, mediated by soluble factors present in young plasma. Mechanistically, the rejuvenating effects of young plasma administration are dependent on the phosphorylation of the transcription factor cAMP response element-binding

protein (CREB) in the aged hippocampus[43]. Further supporting the findings that young blood exposure is beneficial for neuronal function, *in vitro* studies have shown that young mouse serum enhances synaptic density and increases NMDA receptor expression in human excitatory neurons, compared to old serum[61]. Interestingly, administration of human umbilical cord plasma has been shown to increase LTP and improve hippocampal-dependent cognition in aged mice[62], suggesting that young human blood may contain rejuvenating capacity.

These studies have prompted much interest in the identification of anti-aging factors in young plasma for rejuvenaton of the aged hippocampus. Two such factors have been identified to date, tissue inhibitor of metalloproteinases 2 (TIMP2) and osteocalcin (OCN)[60,62]. TIMP2, is elevated in human umbilical cord plasma and decreases in the plasma of mice during aging[62]. Systemic administration of TIMP2 in aged mice promotes LTP and hippocampal-dependent cognition, while depletion of TIMP2 prevents the beneficial effects of human umbilical cord plasma treatment on hippocampal cognition[62]. Similarly, systemic administration of OCN promotes hippocampal cognition in aged mice, while depletion of OCN prevents the rejuvenating effects of young mouse plasma treatment on hippocampal cognition[60].

In addition to these studies demonstrating beneficial effects of pro-youthful soluble factors in blood, recent studies suggest that young hematopoietic cells may exert rejuvenating effects on the aged hippocampus. For example, a recent study employing heterochronic bone marrow transplantation found that reconstituting the blood of aged mice with a young hematopoietic system led to decreased circulating CCL11 and increased cognition[50]. Additionally, the depletion of CD4 T cells or monocytes in the blood of young mice has been shown to impair hippocampal neurogenesis and/or cognition[63–65] suggesting a neurotrophic function of young peripheral immune cells, which may be lost during aging[52].

To date, whether other components of young blood exert rejuvenating effects on the aged hippocampus remains to be fully elucidated. One untested component of young blood with potential rejuvenating capacity, is exosomes. Exosomes, are 40-150 nm vesicles, that carry a

wide array of proteins, mRNAs, and miRNAs[reviewed in 66,67,68]. These vesicles are thought to mediate intercellular communication via fusion with the plasma membrane[69], phagocytosis[70], or via extracellular ligand-receptor signaling[71–73]. Interestingly, emerging studies have increasingly implicated exosomal signaling as a regulator of homeostatic and regenerative capacity in the brain, regulating synaptic plasticity and neurogenesis, *in vitro*[74]. Exosomes are abundant in blood[75], and recent studies suggest that they can cross the blood-brain barrier[76–79] positioning them as a potential link between age-related changes in blood and brain aging. Supporting this hypothesis, a recent study found that young blood-derived exosomes promote myelination in the aged rat motor cortex[80]. Whether young plasma-derived exosomes exert additional rejuvenating effects on the aged brain remains to be tested.

1.4 Aims of this study

The aim of this study is to expand understanding of systemic regulation of hippocampal aging and to identify potential therapeutic strategies to improve cognition in old age.

To that end, in chapter 2, we examine the role of cellular aging of the hematopoietic system in contributing to the pro-aging effects of old blood. We identify the aged hematopoietic system as a driver of the age-related decline in hippocampal neurogenesis, synaptic protein expression, and cognition. At a mechanistic level, we identify a number of aged-hematopoietic system-induced factors, of which Cyclophilin A (CyPA) acts as a pro-aging factor. Systemic upregulation of CyPA impairs hippocampal cognition in young mice, and systemic inhibition of CyPA improves hippocampal cognition in aged mice.

In chapter 3, we examine whether exosomes isolated from young mouse plasma contain rejuvenating capacity in the aged brain. We find that young plasma administration promotes hippocampal neurogenesis, improves hippocampal cognition and increases expression of synaptic proteins in the hippocampus and cortex of aged mice. Additionally, we find that administration of young plasma-derived exosomes does not recapitulate any of the

beneficial effects of young plasma administration on the aged hippocampus. However, aged mice treated with young plasma-derived exosomes exhibited increased synaptic marker expression in cortex, suggesting region specific mechanisms of brain rejuvenation by young blood.

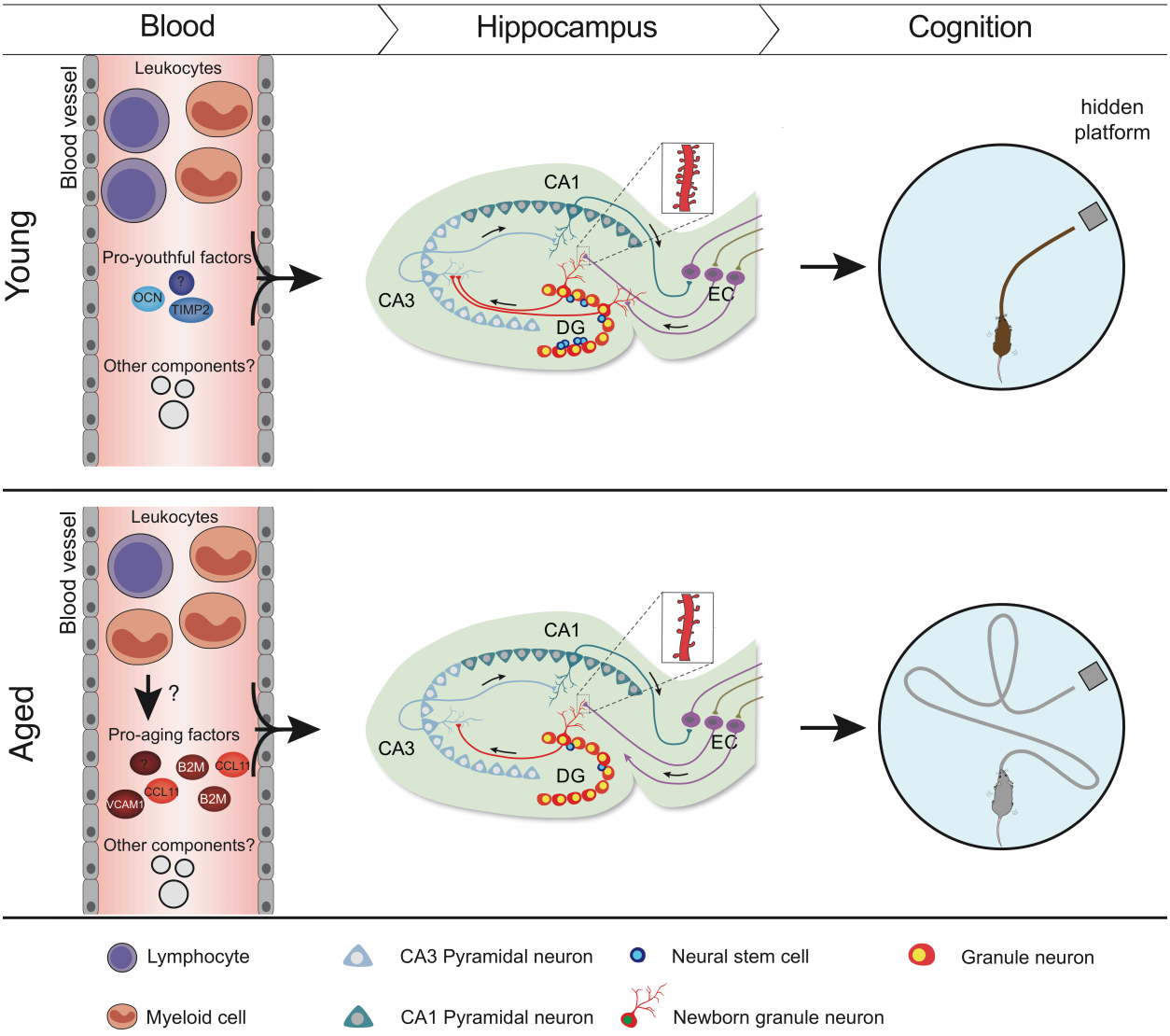


Figure 1.1 Age-related changes in blood regulate hippocampal neurogenesis, neuronal function, and memory. During aging, the cellular and extracellular components of blood change, leading to an accumulation of pro-aging immune molecules and a loss of neurotrophic factors. These changes in blood drive cellular hallmarks of hippocampal aging and impair cognition. To what extent cellular aging of blood contributes to these effects remains to be fully elucidated. Moreover, whether age-related changes in other components of blood, such as exosomes, regulate hippocampal aging remains unknown. Adapted from Smith *et al*, 2018[52] and Fan *et al*, 2017[21].

1.5 References

1. Yun, M. H. Changes in regenerative capacity through lifespan. *International Journal of Molecular Sciences* (2015). doi:10.3390/ijms161025392
2. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* (2013). doi:10.1016/j.cell.2013.05.039
3. Harada, C. N., Natelson Love, M. C. & Triebel, K. L. Normal cognitive aging. *Clinics in Geriatric Medicine* (2013). doi:10.1016/j.cger.2013.07.002
4. Mattson, M. P. & Magnus, T. Ageing and neuronal vulnerability. *Nat. Rev. Neurosci.* **7**, 278–94 (2006).
5. Burgess, N., Maguire, E. A. & O'Keefe, J. The human hippocampus and spatial and episodic memory. *Neuron* (2002). doi:10.1016/S0896-6273(02)00830-9
6. Basu, J. & Siegelbaum, S. A. The corticohippocampal circuit, synaptic plasticity, and memory. *Cold Spring Harbor Perspectives in Biology* (2015). doi:10.1101/cshperspect.a021733
7. Milner, B., Squire, L. R. & Kandel, E. R. Cognitive neuroscience and the study of memory. *Neuron* (1998). doi:10.1016/S0896-6273(00)80987-3
8. Lee, H. K. *et al.* Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* (2003). doi:10.1016/S0092-8674(03)00122-3
9. Niewoehner, B. *et al.* Impaired spatial working memory but spared spatial reference memory following functional loss of NMDA receptors in the dentate gyrus. *Eur. J. Neurosci.* (2007). doi:10.1111/j.1460-9568.2007.05312.x
10. Abbott, L. C. & Nigussie, F. Adult neurogenesis in the mammalian dentate gyrus. *Journal of Veterinary Medicine Series C: Anatomia Histologia Embryologia* (2020). doi:10.1111/ahe.12496
11. Sorrells, S. F. *et al.* Human hippocampal neurogenesis drops sharply in children to

undetectable levels in adults. *Nature* (2018). doi:10.1038/nature25975

12. Spalding, K. L. *et al.* Dynamics of hippocampal neurogenesis in adult humans. *Cell* **153**, 1219–27 (2013).

13. Ernst, A. *et al.* Neurogenesis in the striatum of the adult human brain. *Cell* (2014). doi:10.1016/j.cell.2014.01.044

14. Eriksson, P. S. *et al.* Neurogenesis in the adult human hippocampus. *Nat. Med.* (1998). doi:10.1038/3305

15. Boldrini, M. *et al.* Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell* (2018). doi:10.1016/j.stem.2018.03.015

16. Moreno-Jiménez, E. P. *et al.* Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nat. Med.* (2019). doi:10.1038/s41591-019-0375-9

17. Tobin, M. K. *et al.* Human Hippocampal Neurogenesis Persists in Aged Adults and Alzheimer's Disease Patients. *Cell Stem Cell* (2019). doi:10.1016/j.stem.2019.05.003

18. Deng, W., Saxe, M. D., Gallina, I. S. & Gage, F. H. Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain. *J. Neurosci.* **29**, 13532–13542 (2009).

19. Gu, Y. *et al.* Optical controlling reveals time-dependent roles for adult-born dentate granule cells. *Nat. Neurosci.* **15**, 1700–6 (2012).

20. Berdugo-Vega, G. *et al.* Increasing neurogenesis refines hippocampal activity rejuvenating navigational learning strategies and contextual memory throughout life. *Nat. Commun.* (2020). doi:10.1038/s41467-019-14026-z

21. Fan, X., Wheatley, E. G. & Villeda, S. A. Mechanisms of Hippocampal Aging and the Potential for Rejuvenation. *Annu. Rev. Neurosci* **40**, 251–72 (2017).

22. Arias-Cavieres, A., Adasme, T., Sánchez, G., Muñoz, P. & Hidalgo, C. Aging impairs hippocampal- dependent recognition memory and LTP and prevents the associated RyR

up-regulation. *Front. Aging Neurosci.* (2017). doi:10.3389/fnagi.2017.00111

23. Rosenzweig, E. S., Rao, G., McNaughton, B. L. & Barnes, C. A. Role of temporal summation in age-related long-term potentiation-induction deficits. *Hippocampus* (1997). doi:10.1002/(SICI)1098-1063(1997)7:5<549::AID-HIPO10>3.0.CO;2-0

24. Wheatley, E. G. *et al.* Neuronal O-GlcNAcylation Improves Cognitive Function in the Aged Mouse Brain. *Curr. Biol.* (2019). doi:10.1016/j.cub.2019.08.003

25. Liu, P., Smith, P. F. & Darlington, C. L. Glutamate receptor subunits expression in memory-associated brain structures: Regional variations and effects of aging. *Synapse* (2008). doi:10.1002/syn.20563

26. Shi, L. *et al.* Caloric restriction eliminates the aging-related decline in NMDA and AMPA receptor subunits in the rat hippocampus and induces homeostasis. *Exp. Neurol.* (2007). doi:10.1016/j.expneurol.2007.03.026

27. Dickstein, D. L., Weaver, C. M., Luebke, J. I. & Hof, P. R. Dendritic spine changes associated with normal aging. *Neuroscience* (2013). doi:10.1016/j.neuroscience.2012.09.077

28. Geinisman, Y., De Toledo-Morrell, L. & Morrell, F. Loss of perforated synapses in the dentate gyrus: Morphological substrate of memory deficit in aged rats. *Proc. Natl. Acad. Sci. U. S. A.* (1986). doi:10.1073/pnas.83.9.3027

29. Gontier, G. *et al.* Tet2 Rescues Age-Related Regenerative Decline and Enhances Cognitive Function in the Adult Mouse Brain. *Cell Rep.* (2018). doi:10.1016/j.celrep.2018.02.001

30. Ben Abdallah, N. M. B., Slomianka, L., Vyssotski, A. L. & Lipp, H. P. Early age-related changes in adult hippocampal neurogenesis in C57 mice. *Neurobiol. Aging* (2010). doi:10.1016/j.neurobiolaging.2008.03.002

31. Knoth, R. *et al.* Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PLoS One* (2010). doi:10.1371/journal.pone.0008809

32. Kalamakis, G. *et al.* Quiescence Modulates Stem Cell Maintenance and Regenerative Capacity in the Aging Brain. *Cell* (2019). doi:10.1016/j.cell.2019.01.040

33. Leal, S. L. & Yassa, M. A. Neurocognitive Aging and the Hippocampus across Species. *Trends in Neurosciences* (2015). doi:10.1016/j.tins.2015.10.003

34. Villeda, S. a *et al.* The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* **477**, 90–94 (2011).

35. Kennard, J. A. & Woodruff-Pak, D. S. Age sensitivity of behavioral tests and brain substrates of normal aging in mice. *Front. Aging Neurosci.* (2011). doi:10.3389/fnagi.2011.00009

36. Hedden, T. & Gabrieli, J. D. E. Insights into the ageing mind: a view from cognitive neuroscience. *Nat. Rev. Neurosci.* **5**, 87–96 (2004).

37. Hertzog, C., Dixon, R. A., Hultsch, D. F. & MacDonald, S. W. S. Latent Change Models of Adult Cognition: Are Changes in Processing Speed and Working Memory Associated with Changes in Episodic Memory? *Psychology and Aging* (2003). doi:10.1037/0882-7974.18.4.755

38. Driscoll, I. *et al.* The Aging Hippocampus: Cognitive, Biochemical and Structural Findings. *Cereb. Cortex* (2003). doi:10.1093/cercor/bhg081

39. United Nations, D. of E. and S. A. P. D. World Population Ageing 2013. *2013* (2013). doi:ST/ESA/SER.A/348

40. Ruckh, J. M. *et al.* Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* **10**, 96–103 (2012).

41. Baruch, K. *et al.* Aging-induced type I interferon response at the choroid plexus negatively affects brain function. *Science* **346**, 89–93 (2014).

42. Katsimpardi, L. *et al.* Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science* **344**, 630–4 (2014).

43. Villeda, S. A. *et al.* Young blood reverses age-related impairments in cognitive function

and synaptic plasticity in mice. *Nat. Med.* **20**, 659–63 (2014).

44. Yousef, H. *et al.* Aged blood impairs hippocampal neural precursor activity and activates microglia via brain endothelial cell VCAM1. *Nat. Med.* (2019). doi:10.1038/s41591-019-0440-4

45. Smith, L. K. *et al.* β2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nat. Med.* **21**, 932–7 (2015).

46. Rebo, J. *et al.* A single heterochronic blood exchange reveals rapid inhibition of multiple tissues by old blood. *Nat. Commun.* **7**, 13363 (2016).

47. Targowski, T., Jahnz-Rózyk, K., Plusa, T. & Glodzinska-Wyszogrodzka, E. Influence of age and gender on serum eotaxin concentration in healthy and allergic people. *J. Investig. Allergol. Clin. Immunol.* **15**, 277–282 (2005).

48. Shurin, G. V. *et al.* Dynamic alteration of soluble serum biomarkers in healthy aging. *Cytokine* **39**, 123–129 (2007).

49. Mátrai, Z., Németh, J., Miklós, K., Szabó, Z. & Masszi, T. Serum beta2-microglobulin measured by immunonephelometry: expression patterns and reference intervals in healthy adults. *Clin. Chem. Lab. Med.* **47**, 585–9 (2009).

50. Das, M. M. *et al.* Young bone marrow transplantation preserves learning and memory in old mice. *Commun. Biol.* (2019). doi:10.1038/s42003-019-0298-5

51. Yousef, Hanadie, Conboy, Michael J, Morgenthaler, Adam. Systemic attenuation of the TGF-β pathway by a single drug simultaneously rejuvenates hippocampal neurogenesis and myogenesis in the same old mammal. *Neurobiol. Dis.* **74**, 1–8 (2015).

52. Smith, L. K., White, C. W. & Villeda, S. A. The systemic environment: at the interface of aging and adult neurogenesis. *Cell and Tissue Research* (2018). doi:10.1007/s00441-017-2715-8

53. Wahlestedt, M., Pronk, C. J. & Bryder, D. Concise Review: Hematopoietic Stem Cell Aging and the Prospects for Rejuvenation. *Stem Cells Transl. Med.* **4**, 186–194 (2015).

54. Dorshkind, K., Montecino-Rodriguez, E. & Signer, R. A. J. The ageing immune system: is it ever too old to become young again? *Nat. Rev. Immunol.* **9**, 57–62 (2009).

55. Huang, M.-C., Liao, J.-J., Bonasera, S., Longo, D. L. & Goetzl, E. J. Nuclear factor-kappaB-dependent reversal of aging-induced alterations in T cell cytokines. *FASEB J.* **22**, 2142–50 (2008).

56. Effros, R. B. Role of T lymphocyte replicative senescence in vaccine efficacy. *Vaccine* **25**, 599–604 (2007).

57. Montecino-Rodriguez, E., Berent-Maoz, B. & Dorshkind, K. Causes, consequences, and reversal of immune system aging. *Journal of Clinical Investigation* **123**, 958–965 (2013).

58. Shaw, A. C., Joshi, S., Greenwood, H., Panda, A. & Lord, J. M. Aging of the innate immune system. *Current Opinion in Immunology* **22**, 507–513 (2010).

59. Snyder-Mackler, N., Somel, M. & Tung, J. Shared signatures of social stress and aging in peripheral blood mononuclear cell gene expression profiles. *Aging Cell* **13**, 954–957 (2014).

60. Khrimian, L. *et al.* Gpr158 mediates osteocalcin's regulation of cognition. *J. Exp. Med.* (2017). doi:10.1084/jem.20171320

61. Gan, K. J. & Südhof, T. C. Specific factors in blood from young but not old mice directly promote synapse formation and NMDA-receptor recruitment. *Proc. Natl. Acad. Sci. U. S. A.* (2019). doi:10.1073/pnas.1902672116

62. Castellano, J. M. *et al.* Human umbilical cord plasma proteins revitalize hippocampal function in aged mice. *Nature* **544**, 488–492 (2017).

63. Ziv, Y. *et al.* Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat. Neurosci.* **9**, 268–75 (2006).

64. Wolf, S. A. *et al.* CD4-positive T lymphocytes provide a neuroimmunological link in the control of adult hippocampal neurogenesis. *J. Immunol.* **182**, 3979–84 (2009).

65. Möhle, L. *et al.* Ly6Chi Monocytes Provide a Link between Antibiotic-Induced Changes in

Gut Microbiota and Adult Hippocampal Neurogenesis. *Cell Rep.* 1945–1956 (2016). doi:10.1016/j.celrep.2016.04.074

66. EL Andaloussi, S., Mäger, I., Breakefield, X. O. & Wood, M. J. a. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* **12**, 347–57 (2013).

67. Vlassov, A. V., Magdaleno, S., Setterquist, R. & Conrad, R. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta - General Subjects* **1820**, 940–948 (2012).

68. Roma-Rodrigues, C., Fernandes, A. R. & Baptista, P. V. Exosome in tumour microenvironment: Overview of the crosstalk between normal and cancer cells. *BioMed Research International* **2014**, (2014).

69. Montecalvo, A. *et al.* Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* **119**, 756–766 (2012).

70. Feng, D. *et al.* Cellular internalization of exosomes occurs through phagocytosis. *Traffic* **11**, 675–687 (2010).

71. Gross, J. C., Chaudhary, V., Bartscherer, K. & Boutros, M. Active Wnt proteins are secreted on exosomes. *Nature Cell Biology* **14**, 1036–1045 (2012).

72. Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**, 1161–1172 (1996).

73. Blanchard, N. *et al.* TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J. Immunol.* **168**, 3235–3241 (2002).

74. Saeedi, S., Israel, S., Nagy, C. & Turecki, G. The emerging role of exosomes in mental disorders. *Translational Psychiatry* (2019). doi:10.1038/s41398-019-0459-9

75. Martins, T. S., Catita, J., Rosa, I. M., Da Cruz e Silva, O. A. B. & Henriques, A. G. Exosome isolation from distinct biofluids using precipitation and column-based approaches. *PLoS One* (2018). doi:10.1371/journal.pone.0198820

76. Alvarez-Erviti, L. *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* (2011). doi:10.1038/nbt.1807

77. Chen, C. C. *et al.* Elucidation of Exosome Migration Across the Blood–Brain Barrier Model In Vitro. *Cell. Mol. Bioeng.* (2016). doi:10.1007/s12195-016-0458-3

78. Oberauer, K., Wendland, M. & Kliegl, R. Age differences in working memory - The roles of storage and selective access. *Mem. Cogn.* (2003). doi:10.3758/BF03196097

79. Ridder, K. *et al.* Extracellular Vesicle-Mediated Transfer of Genetic Information between the Hematopoietic System and the Brain in Response to Inflammation. *PLoS Biol.* (2014). doi:10.1371/journal.pbio.1001874

80. Pusic, A. D. & Kraig, R. P. Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination. *Glia* (2014). doi:10.1002/glia.22606

Chapter 2

The aged hematopoietic system promotes hippocampal-dependent cognitive decline

Lucas K. Smith[1,2], Evgenia Verovskaya[3,4], Gregor Bieri[1], Alana M. Horowitz[1,2], Saskia N.I. von Ungern-Sternberg[5], Karin Lin[1,6], Peter Seizer[5], Emmanuelle Passegué[4], and Saul A. Villeda[1,2,3,6]

1. Department of Anatomy, University of California San Francisco, San Francisco, California 94143, USA

2. Biomedical Sciences Graduate Program, University of California San Francisco, San Francisco, California 94143, USA

3. The Eli and Edyth Broad Center for Regenerative Medicine and Stem Cell Research, San Francisco, California 94143, USA

4. Columbia Stem Cell Initiative, Department of Genetics and Development, Columbia University Irving Medical Center, New York, New York 10032, USA.

5. Department of Cardiology and Cardiovascular Medicine, University of Tübingen, Tübingen, Germany

6. Neuroscience Graduate Program, University of California San Francisco, San Francisco, California 94143, USA

2.1 Author Contributions

L.KS. and S.A.V. developed concept and designed experiments. L.K.S. collected and analyzed data. E.V. assisted with HSC transplantation studies and analysis. G.B. and A.M.H. assisted with CyPA-OE studies. S.U-S. and P.S. generated and provided the antibody for CyPA inhibition studies. K.L. assisted with cognitive analysis. E.P. provided reagents and conceptual advice. L.K.S. and S.A.V. wrote manuscript. S.A.V. supervised all aspects of this project.

2.2 Abstract

The aged systemic milieu promotes cellular and cognitive impairments in the hippocampus. Here, we report that aging of the hematopoietic system directly contributes to the pro-aging effects of old blood on cognition. Using a heterochronic hematopoietic stem cell (HSC) transplantation model (in which the blood of young mice is reconstituted with old HSCs) we find that exposure to an old hematopoietic system inhibits hippocampal neurogenesis, decreases synaptic marker expression, and impairs cognition. We identify a number of factors elevated in the blood of young mice reconstituted with old HSCs, of which Cyclophilin A (CyPA) acts as a pro-aging factor. Increased systemic levels of CyPA impair cognition in young mice, while inhibition of CyPA in aged mice improves cognition. Together, these data identify age-related changes in the hematopoietic system as drivers of hippocampal aging.

2.3 Introduction

Exposure to the aging systemic milieu, through models such as heterochronic parabiosis, promotes cellular, molecular, and structural changes in the brain that lead to cognitive decline[1–4]. To date, studies have focused on the role of circulating factors, such as CCL11, B2M, and VCAM-1, as mediators of the pro-aging effects of old blood on the brain[2–5]. Despite the fact that the pro-aging factors identified to date are immune-related molecules[6], whether the aging hematopoietic system promotes hippocampal aging has not yet been investigated.

In mice and humans, the hematopoietic system undergoes many functional and structural changes during aging, characterized by myeloid expansion, decreased immunity, and chronic low grade inflammation[7]. We hypothesized that these cellular changes contribute to hippocampal aging through the accumulation of pro-aging immune factors in old blood.

2.4 Results

Many of the age-related changes observed in old blood have roots in hematopoietic stem cell (HSC) aging[8,9]. Therefore, we employed a heterochronic HSC transplantation model to test how exposure to an aged hematopoietic system contributes to hippocampal aging (**Figure 2.1a**). Young (2 months) recipient mice were sublethally irradiated (9 Gy) and transplanted with HSCs isolated from young (2 months) or old (24 months) donors, generating isochronic (Iso) and heterochronic (Het) HSC reconstituted young mice. Blood chimerism was assessed by measuring the proportion of CD45.2 donor cells in CD45.1 recipient mouse blood by flow cytometry (**Figure 2.1b-c**). Blood derived from old HSCs exhibited characteristic age-related myeloid bias 4.5 months post transplantation (**Figure 2.1d-e**). Animals showed no signs of illness or weight loss regardless of treatment (**Figure 2.1f**).

To investigate whether exposure to an old hematopoietic system elicits age-related cognitive impairments, we assessed hippocampal-dependent associative fear memory and

spatial memory using contextual fear conditioning (FC) and radial arm water maze (RAWM) behavioral paradigms, respectively. While no differences were observed in baseline freezing during the training portion of the FC task (**Figure 2.2a**), Het HSC reconstituted young mice exhibited decreased freezing during contextual FC compared to Iso controls (**Figure 2.2b**), indicating impaired hippocampal-dependent memory. No differences were observed in the amygdala-dependent cued FC paradigm (**Figure 2.2c**). During RAWM, all mice showed similar swim speeds (**Figure 2.2d**) and learning capacity during the training portion of the task (**Figure 2.2e**). Moreover, exposure to an aged hematopoietic system did not result in robust impairments in spatial memory during the testing portion of the task (**Figure 2.2e**). These data indicate that the aged hematopoietic system drives impairments in hippocampal-dependent associative fear memory.

We next sought to assess cellular changes in the hippocampi of Het HSC reconstituted young mice that might contribute to cognitive decline. Hippocampal neurogenesis has been shown to decline with aging and after exposure to an aged systemic milieu[3]. Therefore, we tested whether exposure to an old hematopoietic system impairs neurogenesis by immunohistochemical analysis 4.5 months post HSC transplantation (**Figure 2.3a-b**). Although no difference was observed between groups in the number of Sox2+/GFAP+ neural stem cells in the dentate gyrus (DG), young mice exposed to an old hematopoietic system had decreased numbers of Doublecortin (DCX)-positive immature neurons compared to controls (**Figure 2.3a-b**). To assess neuronal differentiation and survival, we employed a long-term bromodeoxyuridine (BrdU) incorporation paradigm, in which mature neurons derived from proliferating stem cells retain BrdU labeling (**Figure 2.1a**). We observed decreased numbers of BrdU+ cells co-labeled with the mature neuronal marker NeuN in the DG of Het HSC reconstituted young mice compared to Iso controls (**Figure 2.3a-b**). These data indicate that exposure to an old hematopoietic system impairs hippocampal neurogenesis.

We next investigated whether exposure to an aged hematopoietic system elicits synaptic changes in the hippocampus at a molecular and structural level. Activation of the transcription factor, CREB, via phosphorylation, has been implicated in age-related cognitive decline and rejuvenation[10,11]. Correspondingly, we assessed levels of CREB phosphorylation (pCREB) and observed a decrease in Het HSC reconstituted young mice compared to Iso controls (**Figure 2.3c-d**). Moreover, we observed decreased expression of the synaptic markers AMPAR and the NMDA receptor subunit NR2B (**Figure 2.3c-d**), both of which have previously been shown to decline with hippocampal aging[12,13]. At a structural level, we examined dendritic spine density in granule cell neurons, and observed a decrease in Het HSC reconstituted young mice (**Figure 2.3e-f**). Together, these data indicate that the old hematopoietic system leads to an age-related decrease in synaptic density in the hippocampus.

To gain mechanistic insight into how the old hematopoietic system exerts its deleterious effects on cognition, we assessed peripheral immune cell infiltration into the hippocampus. Immunohistochemical identification of CD45.2+ hematopoietic cells in the DG of CD45.1 recipient mice revealed low and equivalent levels of immune cell infiltration in Het and Iso HSC reconstituted young mice (**Figure 2.4a-b**). While we cannot exclude the possible contribution of these small numbers of peripheral immune cells, we hypothesized that the pro-aging effects of the old hematopoietic system is predominantly mediated through peripheral changes in circulating blood factors.

We performed unbiased proteomic analysis on blood plasma collected from Het and Iso HSC reconstituted young mice 4.5 months post transplantation. Using label-free mass spectrometry we identified 22 factors that were differentially expressed between Het and Iso HSC reconstituted young mice (**Figure 2.5a**). To identify potential pro-aging factors, we focused analysis on proteins that were upregulated ≥1.5 fold in the plasma of Het HSC reconstituted young mice (dotted line, **Figure 2.5a**). Of these, the most significantly upregulated cytokine was Cyclophilin A (CyPA, encoded by *Ppia*) - an intracellular protein containing peptidyl-prolyl cis-

trans isomerase activity that is secreted in response to inflammatory stimuli[14]. Elevated CyPA plasma levels were confirmed in Het HSC reconstituted mice by western blot analysis (**Figure 2.5b-c**). In an independent cohort of naïve mice, we detected an age-related increase in CyPA expression in blood cells by qPCR (**Figure 2.5d**). No age-related changes in CyPA expression were observed in other tissues (**Figure 2.5d**). Examination of a previously published human RNA-seq dataset from the GTEx project[15], demonstrated a similar increase of CyPA in human blood cells during aging (**Figure 2.5e**). We next analyzed the relationship between CyPA plasma levels and contextual FC performance in Iso and Het HSC reconstituted mice. We found an inverse correlation between CyPA levels and cognitive performance (**Figure 2.5f**) positing CyPA as a potential pro-aging factor with relevance to cognition.

To test whether increasing systemic CyPA levels is sufficient to elicit age-related cognitive or cellular impairments, we utilized a hydrodynamic tail vein injection (HDTVI) *in vivo* transfection approach, wherein young (2 months) mice were intravenously injected with overexpression constructs encoding either CyPA or GFP control (**Figure 2.6a**). The HDTVI-mediated increase in plasma CyPA levels was confirmed using a HiBiT tagged CyPA and luminescent detection approach (**Figure 2.6b**). Animals showed no signs of illness or weight loss regardless of treatment (**Figure 2.6c**). One month after HDTVI, we assessed hippocampal-dependent object recognition and associative fear memory using novel object recognition (NOR) and contextual FC behavioral paradigms, respectively (**Figure 2.6d-g**). In the testing portion of the NOR task, young control mice exhibited a significant preference for the novel object over the familiar object, consistent with proper cognitive function; however, this preference was lost in young mice overexpressing CyPA (**Figure 2.6d**). In FC, no differences were observed in baseline freezing (**Figure 2.6e**). However, in the hippocampal-dependent contextual FC task, we observed decreased freezing in young mice overexpressing CyPA compared to controls (**Figure 2.6f**). No differences were observed in the amygdala-dependent cued FC task (**Figure 2.6g**). At the cellular level, we examined hippocampal neurogenesis and observed no

differences in the number of DCX-positive immature neurons between treatment groups (**Figure 2.6h-i**). At the molecular level, we assessed synaptic marker expression in the hippocampus of young CyPA overexpressing mice and controls. Increased systemic CyPA resulted in lower levels of NMDA receptor subunit NR2B and the pre-synaptic markers synapsin-1 and synaptophysin compared to control conditions (**Figure 2.6j-k**). These data indicate that increasing systemic CyPA promotes age-related cognitive decline and decreased levels of key proteins important for synapse function.

Last, we explored whether inhibition of systemic CyPA in aged animals could ameliorate cognitive and cellular hallmarks of hippocampal aging. Aged (19 months) mice were administered with an anti-CyPA neutralizing antibody[16] or IgG isotype control, 9 times over one month, via intraperitoneal injection (**Figure 2.7a**). No differences in weight were observed between groups (**Figure 2.7b**). Hippocampal-dependent cognition was examined by NOR and contextual FC behavioral paradigms (**Figure 2.7c-e**). In the testing portion of the NOR task, aged control mice failed to differentiate the novel from familiar object (**Figure 2.7c**), consistent with impaired cognitive function. However, aged mice treated with an anti-CyPA antibody exhibited a significant preference for the novel object (**Figure 2.7c**), indicating improved hippocampal-dependent memory. No differences in freezing were observed in FC (**Figure 2.7d-f**). At the cellular and molecular level, inhibition of systemic CyPA in aged mice increased the number of DCX-positive immature neurons (**Figure 2.7g-h**), and increased expression of the NMDA receptor subunit NR2A and the presynaptic markers synapsin-1 and synaptophysin (**Figure 2.7i-j**). These data indicate that targeting extracellular CyPA at old age is sufficient to improve object recognition memory, promote neurogenesis, and increase the levels of key proteins important for synapse function.

2.5 Discussion

Cumulatively, our data demonstrate that age-related changes in the hematopoietic system promote molecular, cellular, and cognitive hallmarks of hippocampal aging. We identify CyPA as a pro-aging factor whose expression is elevated in the blood of het HSC reconstituted young mice. While the cellular source of CyPA in het HSC reconstituted young mice remains to be elucidated, increasing CyPA in the blood of young mice, by HDTVI mediated overexpression of CyPA, impaired hippocampal-dependent cognition. Moreover, we found that the systemic inhibition of CyPA improved cognition in aged mice. Notably, inhibiting CyPA has been demonstrated to be neuroprotective in a mouse model of Amyotrophic lateral sclerosis[17]. In humans, elevated cerebrospinal fluid CyPA levels has recently been associated with cognitive impairments in Alzheimer's disease patients expressing apolipoprotein E4[18]. Moreover, in humans elevated CyPA plasma levels accompany a number of inflammatory age-related diseases, including diabetes[19] and cardiovascular disease[20,21]. In these studies, CyPA plasma levels were also found to be elevated with aging[19–21]. While little is known about the role of CyPA in aging, recent proteomic analysis using mass spectrometry have identified CyPA as part of the senescence-associated secretory phenotype (SASP)[22]. Ultimately, our data identify the aged hematopoietic system, and downstream circulating immune factors, as potential therapeutic targets to restore cognitive function in the elderly.

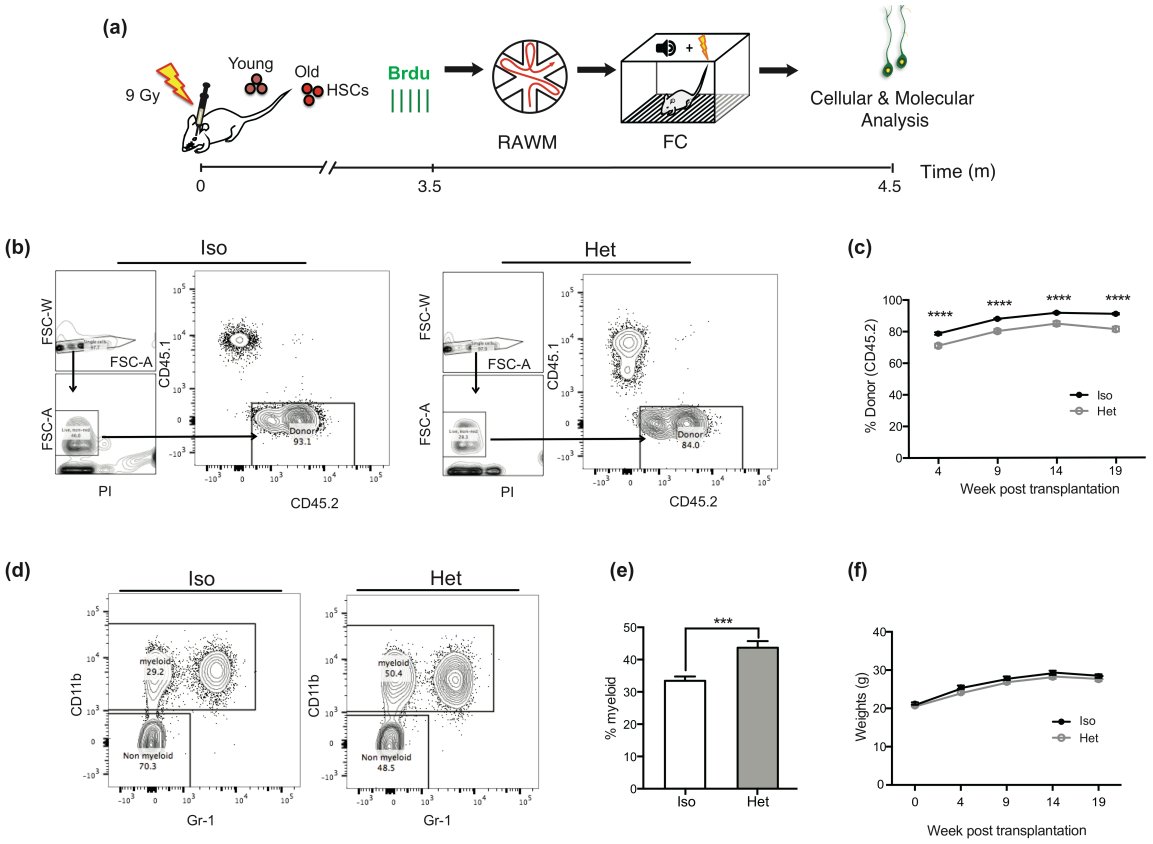


Figure 2.1 Establishment of a heterochronic HSC reconstitution model. (**a**) Schematic showing isochronic (Iso) and heterochronic (Het) HSC transplantation paradigm in young mice. (**b-c**) Representative flow plots (**b**) and quantification (**c**) of peripheral blood chimerism in Iso and Het HSC reconstituted young mice (n=20/group). (**d-e**) Representative flow plot (**d**) and quantification (**e**) of myeloid bias in Iso and Het HSC reconstituted blood, 4.5 mo post transplantation (n=17-19/group). (**f**) Weights of young Iso and Het HSC reconstituted mice (n=14-19/group). All data shown as the mean +*SEM*. ***$p<0.001$, ****<0.0001. Repeated measures analysis of variance (ANOVA) with Sidak correction for multiple comparison (**c,f**). t-test (**e**).

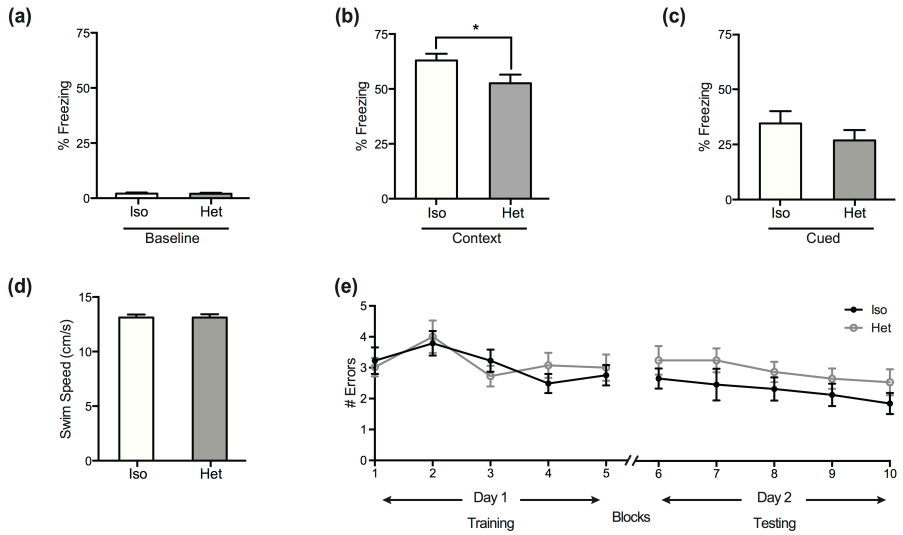


Figure 2.2 The aged hematopoietic system promotes hippocampal-dependent cognitive decline. (**a**) Baseline freezing of isochronic (Iso) and heterochronic (Het) HSC reconstituted young mice during the training portion of fear conditioning (FC) paradigm (n=17-19/group**)**. **(b-c)** Hippocampal- and amygdala- dependent memory was assessed in Iso and Het mice by quantification of freezing during contextual (**b**) or cued (**c**) FC, respectively (n=17-19/group). (**d**) Swim speed of Iso and Het HSC reconstituted young mice during radial arm water maze (RAWM) task (n=17-19/group). (**e**) Number of errors made by Iso and Het HSC reconstituted young mice before finding the hidden platform in the training and testing portions of the RAWM paradigm (n=17-19/group). All data shown as the mean +*SEM*. **p<0.05.* Repeated measures analysis of variance (ANOVA) with Sidak correction for multiple comparison (**e**). t-test (**a-d**).

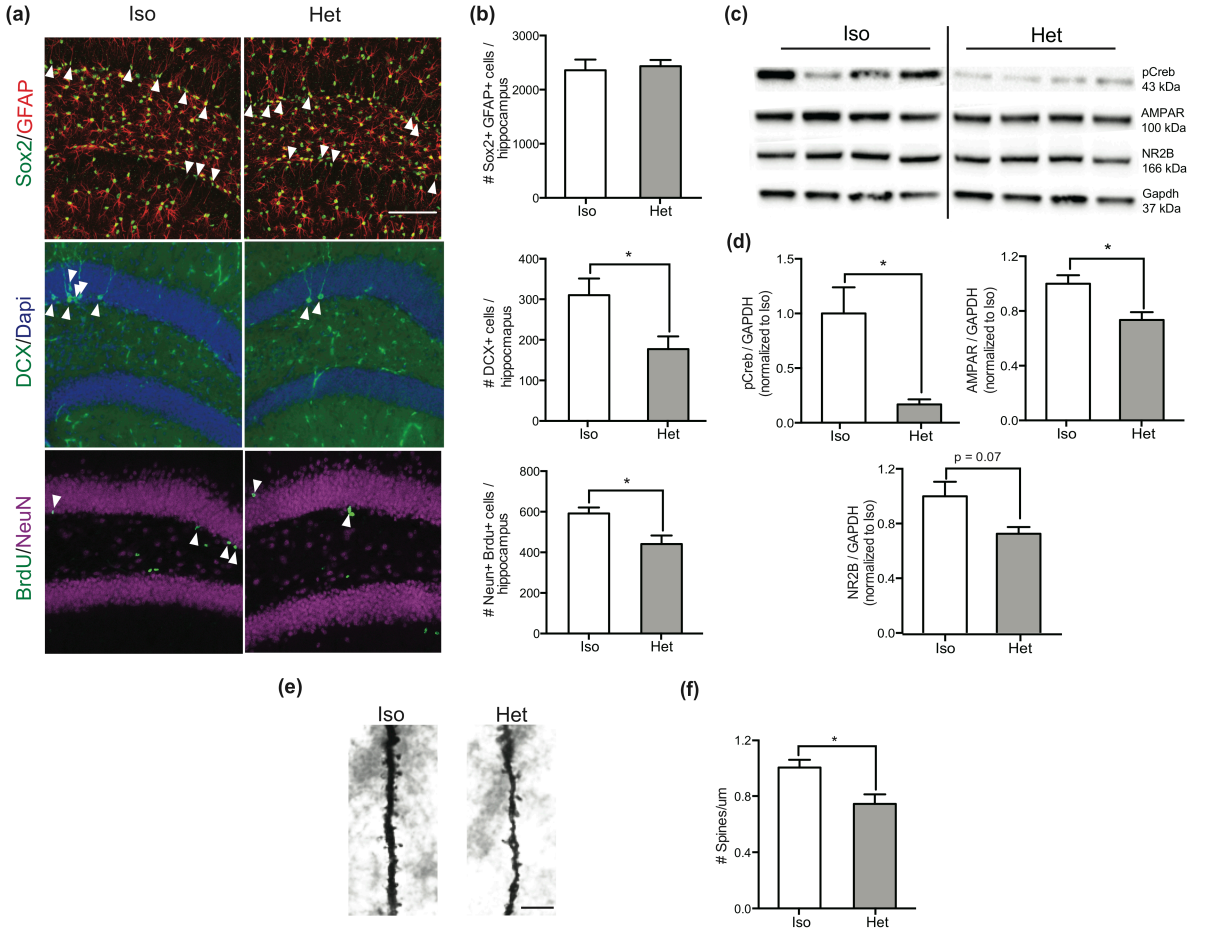


Figure 2.3 The aged hematopoietic system promotes cellular hallmarks of hippocampal aging. (**a-b**) Representative fields (**a**; scale bar=100um**)** and quantification **(b)** of Sox2+/GFAP+ (n=5-8/group), DCX+ (n=7-8/group), and NeuN+/BrdU+ (n=10-13/group) cells in isochronic (Iso) and heterochronic (Het) HSC reconstituted young mice. **(c-d)** Representative western blot **(c)** and quantification (**d**) of hippocampal lysates from Iso and Het mice, probed with anti-pCREB, AMPAR, NR2B, and GAPDH (n=4-5/group). (**e-f**) Representative Golgi stain (**e**; scale bar=5 um) and quantification **(f)** of dendritic spine density on tertiary branches in the dentate gyrus of Iso and Het HSC reconstituted young mice (n=4-5/group)**.** All data shown as the mean +*SEM*. t-test (**b,d,f**).

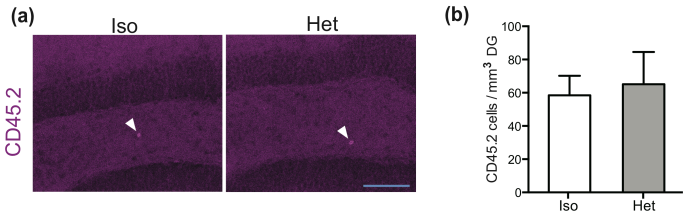


Figure 2.4 Low infiltration of peripheral immune cells in the dentate gyrus of isochronic and heterochronic HSC reconstituted mice. (**a-b**) Representative fields (**a**; scale bar=100 um) and quantification (**b**) of the numbers of CD45.2+ cells observed in the parenchyma of the DG of isochronic (Iso) and heterochronic (Het) HSC reconstituted mice (n=6-7/group). All data shown as the mean +*SEM*. t-test (**b**).

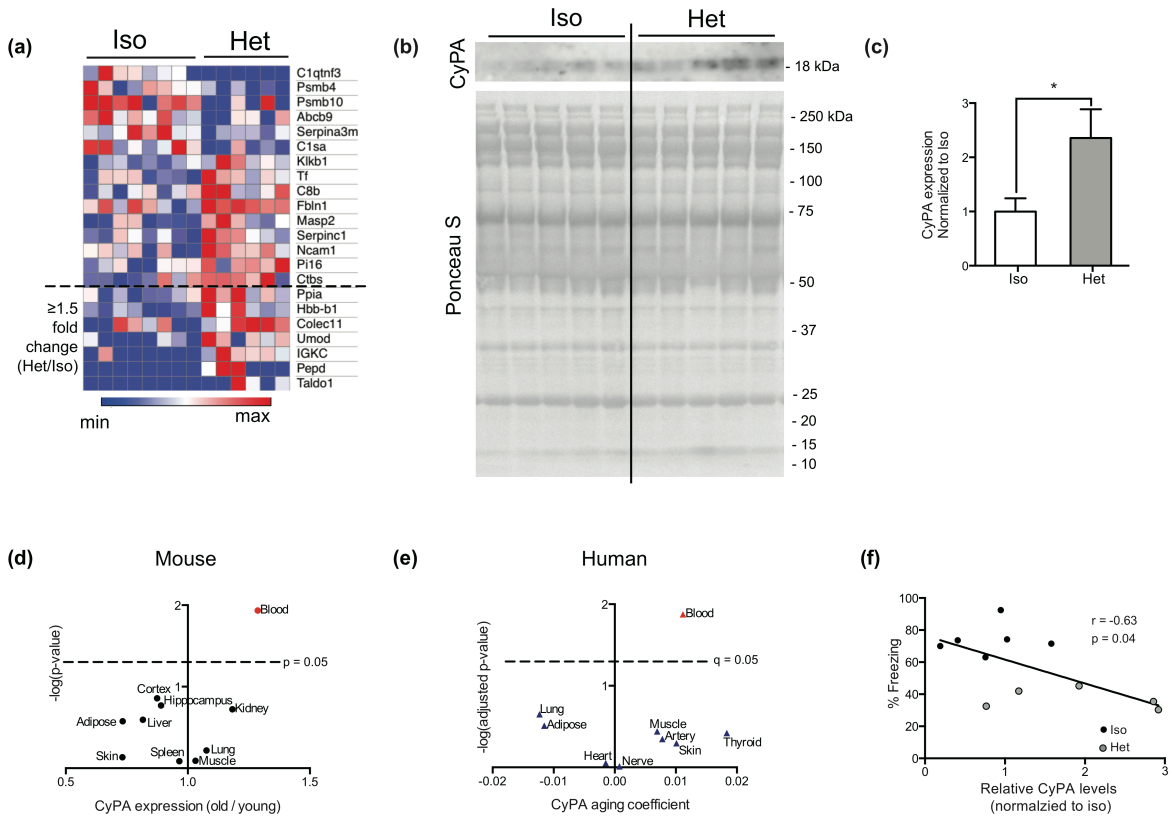


Figure 2.5 Identification of CyPA as a candidate pro-aging factor elevated in the blood of heterchronic HSC reconstituted young mice. (**a**) Heatmap of proteins differentially expressed ($p<0.05$) between plasma of isochronic (Iso) and heterochronic (Het) HSC reconstituted young mice, as determined by label-free mass spectrometry (n=6-8/group). (**b-c**) Representative western blot (**b**) and quantification (**c**) of CyPA in blood plasma of Iso and Het HSC reconstituted young mice. Ponceau S staining shown for loading (n=5-6/group). (**d**) CyPA expression across mouse tissues during aging by qPCR (n=5-6/group). (**e**) Aging-coefficients for CyPA across human tissues from RNAseq dataset (n=83-156 subjects/tissue). (**f**) Correlation of plasma CyPA levels assessed by western blot analysis, with percent time freezing in contextual FC in Iso and Het HSC reconstituted young mice (n=5-6/group). All data shown as the mean +*SEM*. *$p<0.05$. t test (**a,c,d**). Pearson correlation **(f).**

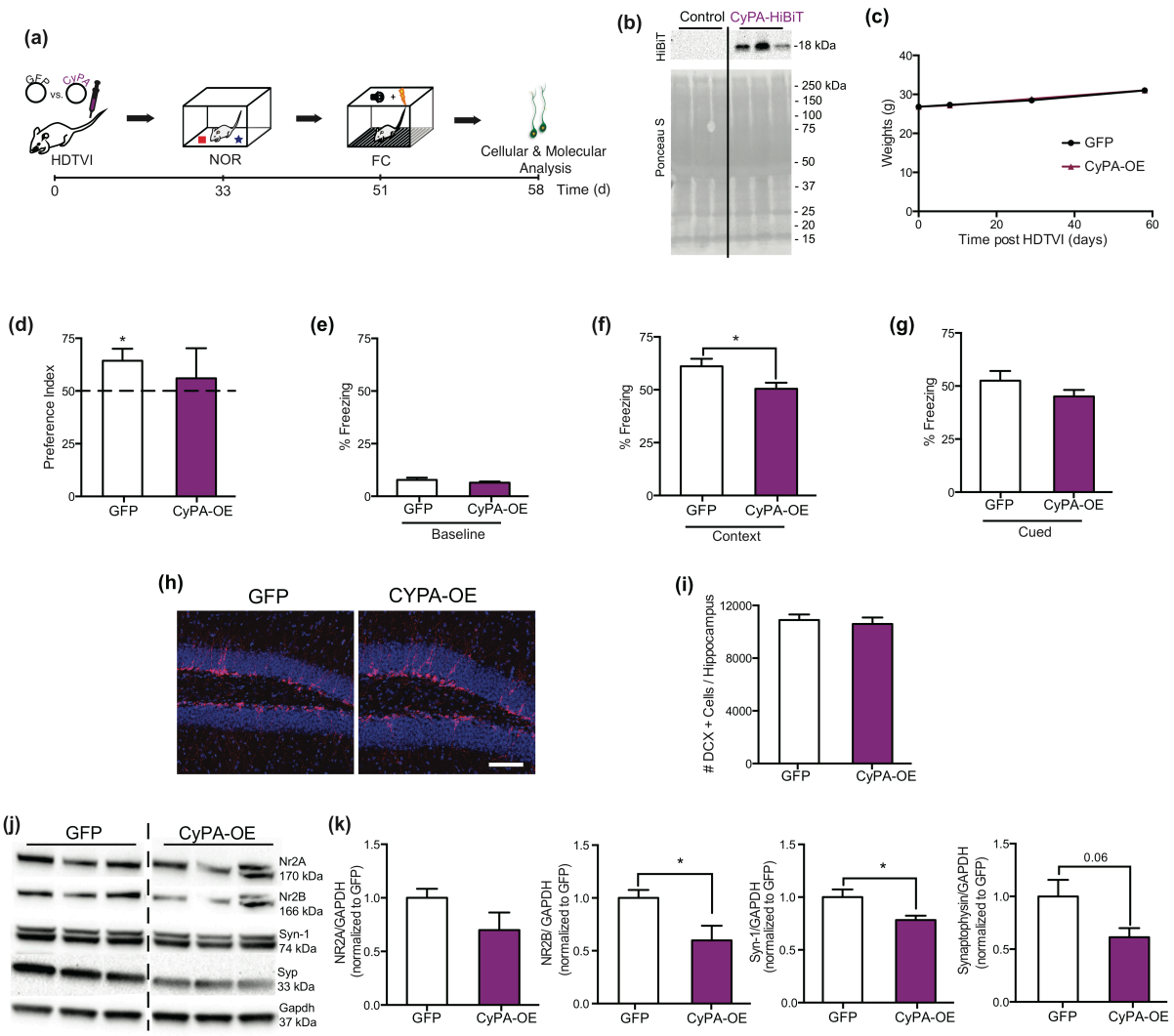


Figure 2.6 Systemic overexpression of CyPA promotes cellular and cognitive hallmarks of hippocampal aging in young mice. (**a**) Schematic showing hydrodynamic tail vein injection (HDTVI)-mediated CyPA overexpression (OE) paradigm in young mice. (**b**) Chemiluminescent detection of HiBiT-tagged-CyPA in blood plasma of CyPA-HiBiT overexpressing mice. Ponceau S staining shown for loading (n=3/group). (**c**) Weights of CyPA-OE and GFP-control young mice (n=16/group). (**d**) Preference of CyPA-OE or GFP mice for the novel object in the testing portion of the novel object recognition (NOR) behavioral paradigm (n=8-12/group). (**e**) Baseline freezing of CyPA-OE or GFP controls during training portion of the fear conditioning (FC) behavioral paradigm (n=15-16/group**)**. **(f-g)** Hippocampal- and amygdala- dependent memory was assessed in CyPA-OE and GFP control mice by contextual FC (**f**; n=15-16) and cued FC (**g**; n=15-16), respectively. (**h-i**) Representative fields **(h**; scale bar=100um**)** and quantification **(i)** of DCX+ cells (n=11/group), in the dentate gyrus of young CyPA-OE and GFP control mice. (**j-k**) Representative western blot **(j)** and quantification (**k**) of hippocampal lysates from young CyPA-OE and GFP control mice, probed with anti-NR2A, anti-NR2B, synapsin-1 (Syn-1), Synaptophysin (Syp), and GAPDH (n=5/group). All data shown as the mean +*SEM*. *$p<0.05$. Repeated measures analysis of variance (ANOVA) with Sidak correction for multiple comparison (**c**). One-sample t test vs. hypothetical mean of 50 (**d**). t test (**e-g,i,k**).

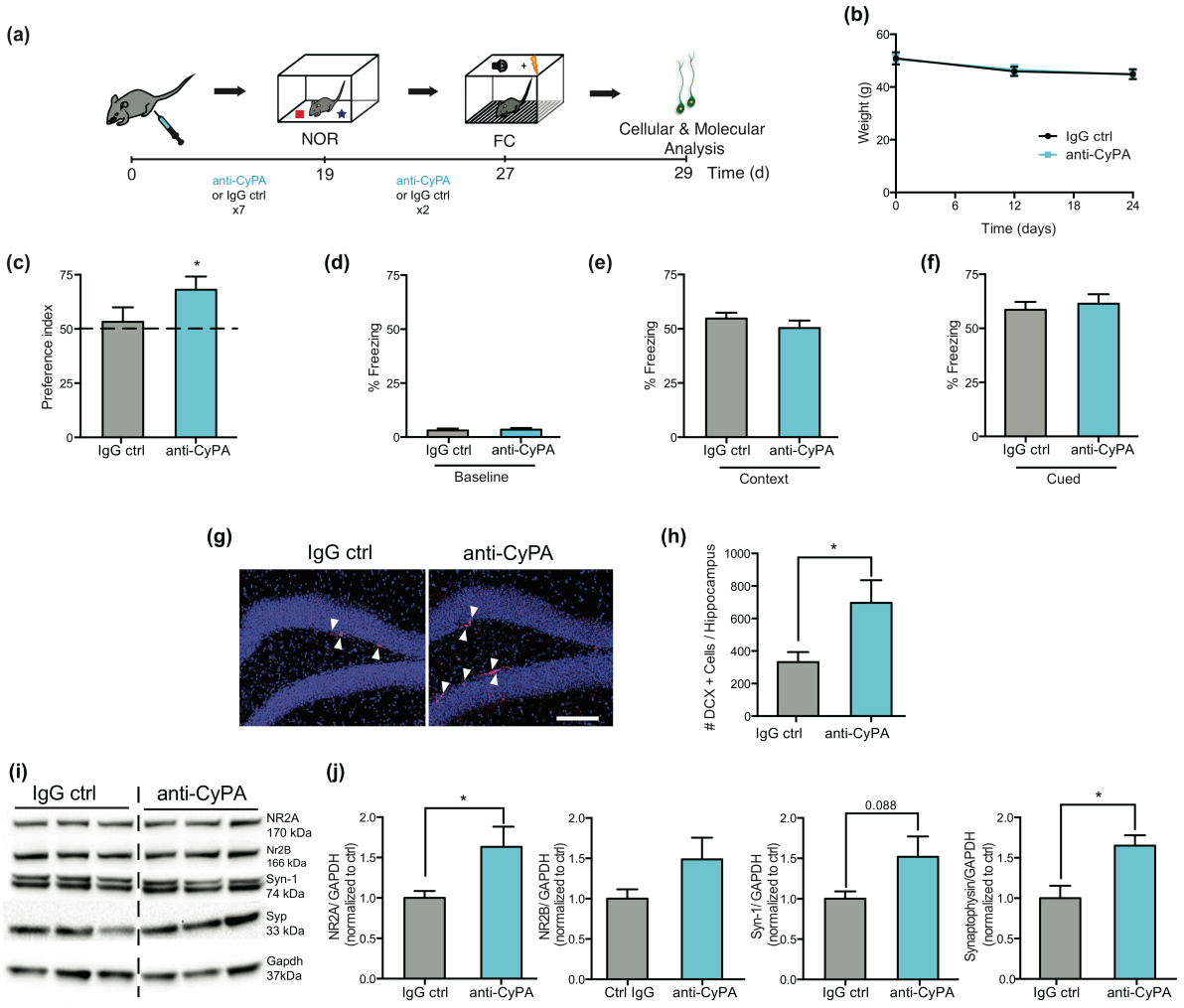


Figure 2.7 Inhibition of systemic CyPA ameliorates cellular and cognitive hallmarks of hippocampal aging. (**a**) Schematic showing CyPA inhibition paradigm in aged mice. (**b**) Weights of aged mice treated with an anti-CyPA or IgG isotope control (ctrl) antibody (n=12-14). (**c**) Preference of aged mice treated with an anti-CyPA or ctrl antibody for the novel object in the testing portion of the novel object recognition (NOR) behavioral paradigm (n=10-14/group). (**d**) Baseline freezing of anti-CyPA or ctrl antibody treated aged mice during training portion of fear conditioning paradigm (n=10-14/group**).** (**e-f**) Hippocampal- and amygdala- dependent memory was assessed in anti-CyPA or ctrl antibody treated aged mice in contextual FC (**e**; n=10-14/group) and cued FC (**f**; n=10-14/group), respectively. (**g-h**) Representative fields **(g**; scale bar=100um**)** and quantification (**h**) of DCX+ (n=8-10/group), in the dentate gyrus of anti-CyPA or ctrl treated aged mice. **(i-j)** Representative western blot **(i)** and quantification (**j**) of hippocampal lysates from aged mice treated with an anti-CyPA or ctrl antibody, probed with anti anti-NR2A, anti-NR2B, synapsin-1 (Syn-1), Synaptophysin (Syp), and GAPDH (n=5/group). Repeated measures analysis of variance (ANOVA) with Sidak correction for multiple comparison (**b**). One-sample t test vs. hypothetical mean of 50 (**c**). t test (**d-f,h,j**).

2.6 References

1. Katsimpardi, L. *et al.* Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science* **344**, 630–4 (2014).
2. Smith, L. K. *et al.* β2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nat. Med.* **21**, 932–7 (2015).
3. Villeda, S. a *et al.* The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* **477**, 90–94 (2011).
4. Yousef, H. *et al.* Aged blood impairs hippocampal neural precursor activity and activates microglia via brain endothelial cell VCAM1. *Nat. Med.* (2019). doi:10.1038/s41591-019-0440-4
5. Das, M. M. *et al.* Young bone marrow transplantation preserves learning and memory in old mice. *Commun. Biol.* (2019). doi:10.1038/s42003-019-0298-5
6. Smith, L. K., White, C. W. & Villeda, S. A. The systemic environment: at the interface of aging and adult neurogenesis. *Cell and Tissue Research* (2018). doi:10.1007/s00441-017-2715-8
7. Van Zant, G. & Liang, Y. Concise Review: Hematopoietic Stem Cell Aging, Life Span, and Transplantation. *Stem Cells Transl. Med.* **1**, 651–657 (2012).
8. Pang, W. W. *et al.* Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 20012–7 (2011).
9. Kim, M., Moon, H. B. & Spangrude, G. J. Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann N Y Acad Sci* **996**, 195–208 (2003).
10. Villeda, S. A. *et al.* Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat. Med.* **20**, 659–63 (2014).
11. Yu, X. W., Curlik, D. M., Oh, M. M., Yin, J. C. P. & Disterhoft, J. F. CREB overexpression in dorsal CA1 ameliorates long-term memory deficits in aged rats. *Elife* (2017).

doi:10.7554/eLife.19358

12. Shi, L. *et al.* Caloric restriction eliminates the aging-related decline in NMDA and AMPA receptor subunits in the rat hippocampus and induces homeostasis. *Exp. Neurol.* (2007). doi:10.1016/j.expneurol.2007.03.026

13. Wheatley, E. G. *et al.* Neuronal O-GlcNAcylation Improves Cognitive Function in the Aged Mouse Brain. *Curr. Biol.* (2019). doi:10.1016/j.cub.2019.08.003

14. Nigro, P., Pompilio, G. & Capogrossi, M. C. Cyclophilin A: A key player for human disease. *Cell Death and Disease* (2013). doi:10.1038/cddis.2013.410

15. Yang, J. *et al.* Synchronized age-related gene expression changes across multiple tissues in human and the link to complex diseases. *Sci. Rep.* (2015). doi:10.1038/srep15145

16. Von Ungern-Sternberg, S. N. I. *et al.* Extracellular cyclophilin a augments platelet-dependent thrombosis and thromboinflammation. *Thromb. Haemost.* (2017). doi:10.1160/TH17-01-0067

17. Pasetto, L. *et al.* Targeting extracellular cyclophilin a reduces neuroinflammation and extends survival in a mouse model of amyotrophic lateral sclerosis. *J. Neurosci.* (2017). doi:10.1523/JNEUROSCI.2462-16.2016

18. Montagne, A. *et al.* APOE4 leads to blood–brain barrier dysfunction predicting cognitive decline. *Nature* (2020). doi:10.1038/s41586-020-2247-3

19. Ramachandran, S. *et al.* Plasma level of cyclophilin A is increased in patients with type 2 diabetes mellitus and suggests presence of vascular disease. *Cardiovasc. Diabetol.* (2014). doi:10.1186/1475-2840-13-38

20. Satoh, K. *et al.* Plasma cyclophilin a is a novel biomarker for coronary artery disease. *Circ. J.* (2013). doi:10.1253/circj.CJ-12-0805

21. Ohtsuki, T. *et al.* Prognostic impacts of plasma levels of cyclophilin a in patients with coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* (2017).

doi:10.1161/ATVBAHA.116.308986

22. Wiley, C. D. *et al.* SILAC Analysis Reveals Increased Secretion of Hemostasis-Related Factors by Senescent Cells. *Cell Rep.* (2019). doi:10.1016/j.celrep.2019.08.049
23. Ho, T. T. *et al.* Autophagy maintains the metabolism and function of young and old stem cells. *Nature* (2017). doi:10.1038/nature21388
24. Ardlie, K. G. *et al.* The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue gene regulation in humans. *Science (80-.).* (2015). doi:10.1126/science.1262110
25. Kovacsics, D. & Raper, J. Transient expression of proteins by hydrodynamic gene delivery in mice. *J. Vis. Exp.* (2014). doi:10.3791/51481
26. Alamed, J., Wilcock, D. M., Diamond, D. M., Gordon, M. N. & Morgan, D. Two-day radial-arm water maze learning and memory task; robust resolution of amyloid-related memory deficits in transgenic mice. *Nat. Protoc.* (2006). doi:10.1038/nprot.2006.275
27. Dubal, D. B. *et al.* Life extension factor klotho prevents mortality and enhances cognition in hAPP transgenic mice. *J. Neurosci.* (2015). doi:10.1523/JNEUROSCI.5791-12.2015
28. Tyanova, S., Temu, T. & Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat. Protoc.* (2016). doi:10.1038/nprot.2016.136
29. Geyer, P. E. *et al.* Plasma Proteome Profiling to Assess Human Health and Disease. *Cell Syst.* (2016). doi:10.1016/j.cels.2016.02.015

2.7 Materials and Methods

Mice. Young (2-months) and old (19-24 mo) wild-type C57BL/6J and B6.SJL/BoyJ mice were obtained from the Jackson Laboratory and subsequently maintained in-house under specific pathogen-free conditions under a 12-h light-dark cycle. All studies were done in male mice. The numbers of mice used to result in statistically significant differences was calculated using standard power calculations with $\alpha = 0.05$ and a power of 0.8. We used an online tool (http://www.stat.uiowa.edu/~rlenth/Power/index.html) to calculate power and sample size on the basis of experience with the respective tests, variability of the assays and inter-individual differences within groups. All animal handling and use was in accordance with institutional guidelines approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC).

Irradiation and HSC transplantation. For transplantation studies, 2-month-old CD45.1 C57Bl/6-Boy/J recipient mice were sub-lethally irradiated (9 Gy, administered in split doses, 3 hours apart) using a ^{137}Cs source (J.L. Shepherd). 2000 purified CD45.2 C57BL/6J HSCs were transplanted by retro-orbital injection to recipient mice, within 24h of irradiation. Recipient mice were maintained on antibiotic-containing water for 4 weeks post transplantation. Blood chimerism was assessed every 5 weeks by retro-orbital bleeding and flow cytometry. Mice with <80% blood chimerism, 3 months-post transplantation, were excluded from further analysis.

Flow cytometry. HSCs were isolated as previously described[23]. Bone marrow was obtained from leg, arm, and pelvic bones, isolated in Hanks' buffered saline solution (HBSS) supplemented with 2% heat-inactivated FBS. Erythrocytes were lysed in ACK (150 mM NH4Cl/10 mM kHCO3/10 mM EDTA). Bone marrow cells were further purified using a Ficoll gradient (Histopaque 119, Sigma-Aldrich). c-Kit-positive cells where enriched for with c-Kit microbeads (Miltenyi Biotec) and MACS Separation LS columns (Miltenyi Biotec). Bone marrow

cells were then incubated with purified rat anti-mouse antibodies for CD3 (BioLegend), B220 (eBioscience), CD4 (eBioscience), CD5 (eBioscience), CD8 (eBioscience), CD11b (eBioscience), GR-1 (eBioscience), Ter119 (eBioscience), followed by goat-anti-rat-PE-Cy5 (Invitrogen). Cells were blocked with purified rat IgG (Sigma-Aldrich) and stained with c-Kit-APC-eFluor780 (eBioscience), Sca-1-PB (BioLegend), CD48-A647 (BioLegend), CD150-PE (BioLegend), and Flk2-bio (eBioscience), followed by SA-PE-Cy7 (eBioscience). Stained bone marrow cells were re-suspended in 1 ug/ml propidium iodine (PI) to exclude dead cells. HSCs were isolated by double sorting with a FACSAria II (BD Biosciences). For blood chimerism analysis, erythrocytes were lysed in ACK, and cells were maintained in HBSS + 2% heat-inactivated FBS. Blood was stained with CD11b-PE-Cy7 (eBioscience), Gr1-PB (eBioscience), B220 APC-Cy7 (eBioscience), CD3 APC (eBioscience), and TER119-PE-Cy5 (eBioscience), CD45.1-PE (eBioscience) and CD45.2-FITC (eBioscience). Stained blood cells were re-suspended in 1 ug/ml PI (Thermo Fischer) to exclude dead cells. Chimerism was analyzed with a FACS LSR II using DIVA software (BD Biosciences)

BrdU administration. For long-term BrdU labeling studies, 50 mg/kg of BrdU (Sigma-Aldrich) was injected intraperitoneally into mice daily for 5 days. Mice were euthanized 33-days after first BrdU injection.

Immunohistochemistry. Tissue processing and immunohistochemistry was performed on free-floating sections according to standard published techniques [2]. Mice were anesthetized with 87.5 mg/kg ketamine and 12.5mg/kg xylazine and transcardially perfused with 1x phosphate buffered saline (PBS). Brains were removed and fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4^{o}C for 48 h before cryoprotection in 30% sucrose. Brains were sectioned coronally, at 40 uM with a cryomicrotome (Leica Camera, Inc.) and stored in cryoprotective medium. Primary antibodies used were: goat anti-DCX (1:7000; Santa Cruz), rat

anti-BrdU (1:1000; Abcam), mouse anti-NeuN (1:1000; Millipore), goat anti-Sox2 (1:200, Santa Cruz), rabbit anti-GFAP (1:1000, Dako), ant-CD45.2-Alexa Fluor 647 (BioLegend). After overnight incubation, unconjugated primary antibody staining was revealed using fluorescence-conjugated secondary antibodies (Life Technologies). For BrdU labeling, brain sections were pre-treated with 2 N HCl at 37^{o}C for 30 min, and washed three times with Tris-buffered Saline with Tween (TBST) before incubation with primary antibody. All cells were counted in the DG of every sixth coronal hemibrain section through the hippocampus and analyzed by confocal or epifluourescent microscopy using a Zeiss Axio Observer.Z1 or Axio Imager M2 microscope, respectively. CD45.2 cells were imaged as a Z-stack and counted in a standardized box centered on the DG and covered part of the hilus, granular cell layer, and molecular layer. CD45.2 counts were normalized to volume.

Golgi Staining and analysis. Mice were anesthetized with 87.5 mg/kg ketamine and 12.5mg/kg xylazine and brains were removed without perfusion. Golgi staining was performed using FD Rapid GolgiStain Kit (FD Neurotechnologies, Inc.; PK401), per manufacturers instructions. Hemibrains were rinsed in Milli-Q water and immersed in the impregnation solution (FD Solutions A+B) for 2 weeks (replacing impregnation solution one time, 24 hours after immersion). Samples were transferred into FD Solution C and incubated for 1 week, (replacing FD Solution C one time, 24 hours after immersion). 200 um sections were cut using a cryomicrotome (Leica Camera, Inc.), and sections were mounted on gelatin-coated slides (FD NeuroTechnologies) in a drop of FD Solution C. After drying, slides were rinsed in Milli-Q H2O 2x for 4 min. Slides were transferred to developing solution (FD Solution D+E) for 10 min. Slides were then rinsed 2x in Milli-Q water, 4 min each, and dehydrated in 50%, 75%, 95%, (1x4 min each), and 100% ETOH (4x 4 min). Slides were then incubated in xylene 3x, 4 min each and coversliped with Permount (Fisher). Tertiary dendritic spines on granule cell neurons were imaged at 63x. Spines were counted on 2-3 tertiary dendrites/neuron and 5-11 neurons/mouse.

Western blot analysis. Mouse hippocampi were dissected after perfusion of animals, snap frozen, and lysed in RIPA lysis buffer (500 nM Tris, pH7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and complete protease inhibitors; Roche). For analysis of synaptic genes, in the Iso and Het HSC reconstituted cohort and GFP- or CyPA- overexpression cohort, mice with >55% contextual freezing in the control group and <55% contextual freezing in the treatment group were used. For analysis of plasma CyPA levels in Iso and Het mice, samples were depleted of the most abundant proteins using Proteome Purify 2 Mouse Serum Protein Immunodepletion Resin (R&D Systems) according to the manufacturer's protocol. For analysis of HiBiT-tagged-CyPA in plasma of HDTVI mice, equal volumes of blood plasma were used. Samples were mixed with 4x NuPage LDS loading buffer (Invitrogen) and loaded on a 4-12% SDS polyacrylamide gradient gel (Invitrogen) and subsequently transferred onto a nitrocellulose membrane. The blots were blocked in 5% milk in TBST and incubated with rabbit anti-pCREB (1:1000; Cell Signaling), rabbit anti-AMPAR (1:3000; Abcam), mouse anti-Synaptophysin (1:1000; Millipore), rabbit anti-NR2B (1:2000; Millipore), rabbit anti-NR2A (1:4000;Millipore), rabbit anti-synapsin-1 (1:5000; Abcam), mouse anti-GAPDH (1:5000; Abcam), and rabbit anti-CyPA (1:200; Enzo Life Sciences). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) kit (GE Healthcare) were used to detect protein signals. Membranes were imaged using a Chemidoc system (BioRad) and quantified using ImageJ software (Version 1.52a). GAPDH bands were used for normalization for hippocampal lysates. Equal loading of plasma was confirmed using Ponceau S solution (Sigma-Aldrich). HiBiT was detected using the Nano-Glo HiBit lytic detection system (Promega) and luminescence was recorded with Chemidoc (BioRad).

qPCR. Tissue was dissected, snap frozen, and total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was subsequently synthesized and mixed using the HighCapacity cDNA

Reverse Transcription kit (ThermoFisher Scientific, Cat# 4374966) and mixed SYBR Fast mix (Kapa Biosystems), and our primers of interest. Quantitative RT-PCR was carried out in a CFX384 Real Time System (Bio-Rad). The following primers were used: GAPDH (F-AGGTCGGTGTGAACGGATTTG; R- TGTAGACCATGTAGTTGAGGTCA), PPIA (F-GGCAAATGCTGGACCAAAC; R- CATTCCTGGACCCAAAACG).

Tissue expression of CyPA (human). Yang et al, (2015) identified age-related gene expression changes in human RNAseq data collected by the Genotype-Tissue Expression (GTEx) project[24]. They examined tissue expression changes in nine tissues: subcutaneous adipose, tibial artery, left ventricle heart, lung, skeletal muscle, tibial nerve, skin, thyroid, and whole blood (n/tissue = 83-156 subjects, ages 20-70 years). Using a linear regression model, Yang *et al.* generated an aging coefficient for each gene detected in each tissue. Aging coefficients that are significantly >0 after false discovery rate adjustments ($q<0.05$) are upregulated with age. We examined how CyPA expression changes with age in their dataset by graphing the aging coefficient of CyPA against the –log10 adjusted q-value.

Plasmid generation. RNA was isolated from adult mouse spleen tissue using TRIZol reagent (Thermo Fisher Scientific) and PureLink™ RNA Mini Kit following the manufacturer's instructions. The RNA concentration was determined via Nanodrop and RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and oligo dT primers (Promega). The following primers were used for PCR amplification of the *PPIA* coding sequences and partial 3' and 5' untranslated regions (UTRs) from cDNA: CACCATGGTCAACCCCACCGTGTT (forward primer); GGTAAAATGCCCGCAAGTCA (reverse primer). The *PPIA* PCR product was purified, cloned into the pENTR D-TOPO vector (ThermoFisher Scientific) and sequence verified using standard M13F, M13R primers. The

coding sequence was further amplified and cloned into a bicistronic mammalian expression plasmid using the restriction sites NheI and EcoRI. A canonical Kozak sequence (GCCACC) was included in the forward primer. For the C-terminal HiBiT tagged PPIA, the nucleotide sequence coding for the 11amino acid HiBiT tag was include in the reverse primer. The resulting bicistronic plasmid vectors expressed untagged or HiBiT-tagged Ppia and an IRES eGFP reporter using a CMV promoter. An empty IRES eGFP construct, based on the same plasmid, was used as a control for *in vivo* experiments. All coding sequences of the plasmids were verified by Sanger sequencing. Endotoxin free plasmid kits were used for plasmid preparation prior to in vivo use.

Hydrodynamic tail vein injection. The hydrodynamic tail vein injection protocol was adapted from a previously described protocol[25]. Endotoxin-free plasmids were prepared using the Qiagen Maxi-Prep Plus Kit (VWR). CyPA, CyPA-HiBiT, or GFP plasmid DNA (50ug) was suspended in 10% body weight saline and injected in the tail vein in 5-7 seconds in young mice. For detection of HiBiT-tagged CyPA, blood was drawn 24h post HDTVI. Following a similar timeline as previous pro-aging studies[2], behavioral testing was started 33 days post HDTVI.

CyPA neutralizing antibody administration. The anti-CyPA antibody, 8H7[16], or a monoclonal IgG2A isotype control (R&D Systems) was intraperitoneally administered to aged (19-months) mice (20ug/kg). Prior to behavioral testing antibodies were administered 7 times every third day, with two additional injections administered between behavioral testing.

Radial arm water maze. Spatial learning and memory was assessed using the radial arm water maze (RAWM) paradigm according to established protocol [26]. In this task the location of the goal arm, which contains a platform, remains constant throughout the training and testing phase, while the start arm is changed during each trial. On day one during the training phase,

mice are trained for 15 trails, with trials alternating between a visible and hidden platform. On day two during the testing phase, mice are tested for 15 trials with a hidden platform. Entry into an incorrect arm is scored as an error, and errors are averaged over training blocks (three consecutive trials).

Contextual fear conditioning. In this task, mice learned to associate the environmental context (fear conditioning chamber) with an aversive stimulus (mild foot shock; unconditioned stimulus, US) enabling testing for hippocampal-dependent contextual fear conditioning. As contextual fear conditioning is hippocampus and amygdala dependent, the mild foot shock was paired with a light and tone cue (conditioned stimulus, CS) in order to also assess amygdala-dependent cued fear conditioning. Conditioned fear was displayed as freezing behavior. Specific training parameters are as follows: tone duration is 30 s; level is 70 dB, 2 kHz; shock duration is 2 s; intensity is 0.6 mA. This intensity is not painful and can easily be tolerated but will generate an unpleasant feeling. On day 1 each mouse was placed in a fear-conditioning chamber and allowed to explore for 2 min before delivery of a 30-s tone (70 dB) ending with a 2-s foot shock (0.6 mA). Two minutes later, a second CS-US pair was delivered. On day 2, each mouse was first placed in the fear-conditioning chamber containing the same exact context, but with no CS or foot shock. Freezing was analyzed for 2 min. One hour later, the mice were placed in a new context containing a different odor, cleaning solution, floor texture, chamber walls and shape. Animals were allowed to explore for 2 min before being re-exposed to the CS. Freezing was analyzed for 30s after re-exposure to CS. Freezing was measured using a FreezeScan video tracking system and software (Cleversys, Inc) or EthoVision XT 11.5 tracking software (Noldus) and a Ugo Basile FC system.

Novel object recognition. The novel object recognition task was adapted from a previously described protocol[27]. During the habituation phase (day 1), mice could freely explore an empty

arena for 10 minutes. During the training phase (day 2), mice were exposed to two identical objects (either two striped scintillation vials or two lego constructions). Mice were allowed to explore the objects for 5 minutes. Mice that did not explore the familiar objects for more than 3s during the training phase were excluded from analysis. During testing (day 3), one familiar object was replaced with a novel object (vial replaced with lego or lego replaced with vial), and mice could explore for 5 minutes. Time spent exploring each object was quantified using the Smart Video Tracking Software (Panlab; Harvard Apparatus).

Plasma collection. At time of euthanasia, mouse blood was collected via intracardial bleeds into EDTA coated tubes. Plasma was generated by centrifugation of freshly (<30 min) isolated blood at 1,000g. Aliquots were stored at -80^{o}C until use.

Label-Free Mass Spectrometry. For proteomic analysis of old-hematopoietic system associated factors, plasma was collected from young Iso and Het HSC reconstituted that had exhibited >60% or <50% freezing in contextual FC, respectively. Plasma was collected from 8 individual mice per condition for a total of 16 samples. Samples were depleted of the most abundant proteins using Proteome Purify 2 Mouse Serum Protein Immunodepletion Resin (R&D Systems) according to the manufacturer's protocol. Depleted samples were buffer exchanged into water on a Corning Spin X 5kD molecular weight cut off spin column and quantified by Qubit fluorometry (Life Technologies). Plasma proteins (50µg) were reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin (Promega). Individual digested samples were processed by solid phase extraction using an Empore C18 (3M) plate under vacuum. Briefly, columns were activated (400µL 95% acetonitrile/0.1% TFA X2) and equilibrated (400µL 0.1% TFA X4). Next, acidified samples were loaded and columns were washed (400µL 0.1% TFA X2). Finally, peptides were eluted (200µL 70% acetonitrile/0.1% TFA X2) and lyophilized for downstream processing. For mass spectrometry analysis, 2µg of protein per sample was

analyzed by nano LC-MS/MS with a Waters NanoAcquity interfaced to a ThermoFisher Fusion Lumos mass spectrometer. Peptides were loaded on a trapping column and eluted over a 75 µm x 50cm analytical column (ThermoFisher P/N ES-803) at 300nL/min using a 3-hour reverse phase gradient. The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 15,000 FWHM for MS and MS/MS respectively. The instrument was run with a 3s cycle for MS and MS/MS and APD was enabled. The data were processed with MaxQuant (version 1.6.0.13; Max Planck Institute for Biochemistry; [28], which incorporates the Andromeda search engine. Using this program, the MS data were recalibrated, protein/peptide identification was made using the Andromeda database search engine, the database search results were filtered at the 1% protein and peptide FDR, and protein levels were quantified. The resulting MaxQuant output was further processed using Perseus (V 1.6.0.7; Max Planck Institute for Biochemistry). Two samples exhibiting clotting changes as described in Geyer et al., 2016[29] were excluded from analysis. Differentially expressed genes were identified by comparing LFQ intensities for each protein detected in Heterochronic HSC reconstituted mice compared to Isochronic HSC reconstituted controls.

Data and statistical analysis. Mice were randomized prior to treatment. Researchers were blinded throughout histological, biochemical and behavioral assessments. Groups were un-blinded at the end of each experiment upon statistical analysis. Data are expressed as mean +*SEM*. The distribution of data in each set of experiments was tested for normality using D'Agostino-Pearson omnibus test or Shapiro-Wilk test. No significant differences in variance between groups were detected using an F test. Statistical analysis was performed with Prism 6.0 software (GraphPad Software). Means between two groups were compared with two-tailed, unpaired Student's *t*-test. Comparisons of means from multiple groups with each other or against one control group were analyzed with one-way ANOVA followed by

appropriate *post hoc* tests (indicated in figure legends). The data that support the findings of this study are available from the corresponding author upon reasonable request.

Chapter 3

Young plasma and young plasma-derived exosomes differentially affect brain aging in a regionally specific manner

Lucas K. Smith[1,2], Xuelai Fan[1,3], Saul A. Villeda[1,2,3]

1. Department of Anatomy, University of California San Francisco, San Francisco, California 94143, USA

2. Biomedical Sciences Graduate Program, University of California San Francisco, San Francisco, California 94143, USA

3. The Eli and Edyth Broad Center for Regenerative Medicine and Stem Cell Research, San Francisco, California 94143, USA

3.1 Author Contributions

L.K.S, X.F., and S.A.V. developed concept and designed experiments. L.K.S. and X.F. collected and analyzed the data. L.K.S. and S.A.V. wrote manuscript. S.A.V. supervised all aspects of this project.

3.2 Abstract

Aging drives impairments in hippocampal neurogenesis, synaptic function, and cognition. Recent studies have revealed that systemic exposure to young blood plasma rejuvenates neuronal and cognitive hallmarks of hippocampal aging. However, which components of young plasma contain rejuvenating capacity remains to be fully elucidated. Here, we characterize the rejuvenating effects of young plasma on hippocampal-dependent cognition, neurogenesis, and synaptic marker expression, and investigate the role of exosomes as a potential mediator of the beneficial effects of young plasma administration on brain aging. While we observe increased hippocampal neurogenesis and synaptic marker expression, accompanied by corresponding improvements in cognition in aged mice treated with young plasma, administration of young plasma-derived exosomes, at physiological levels, did not affect hippocampal aging phenotypes. We additionally examined whether the rejuvenating effects of young plasma extend to other brain regions, and found that young plasma administration led to increased synaptic marker expression in the aged cortex. Interestingly, we found that treatment with young plasma-derived exosomes similarly elicited increased synaptic marker expression in the cortex of aged mice. Cumulatively, our data indicate that young plasma administration exerts broad-ranging rejuvenating effects on the aging brain, and that young plasma-derived exosomes may contribute to this rejuvenation in a region specific manner.

3.3 Introduction

Aging is associated with impaired cognitive ability and increased risk for neurodegenerative disease[1,2]. Particularly sensitive to the effects of aging are functions governed by the cerebrum, and indeed a marked decline in hippocampal- and cortical-dependent episodic and working memories have been observed in rodents, monkeys, and humans[3,4]. While the mechanisms by which age-related cognitive impairments emerge are multifaceted, principal hallmarks of brain aging underlying cognitive decline include neuronal dysfunction and decreased plasticity. In the hippocampus, this is manifested, by a loss of neurogenesis, decreased synaptic density, and decreased expression of synaptic plasticity related molecules[5,6]. Similarly, in the cortex, synaptic loss has been observed during aging with corresponding decreases in the expression of genes important for synaptic function[3,7].

A strategy that has emerged to counteract these changes is treatment with young blood[8–11]. Exposure to young blood, in the model of heterochronic parabiosis, has been shown to improve hippocampal plasticity, leading to increased neurogenesis and increased long-term potentiation in hippocampal slices[8,9]. Notably, the benefits of young blood treatment are, at least in part, mediated by the non-cellular component of blood, as treatment with young mouse or human plasma has been shown to increase hippocampal synaptic plasticity and cognition in aged mice[9,10,12]. At a mechanistic level, research has focused on the effects of soluble blood-borne factors as mediators of the rejuvenating effects of young blood exposure on the aged brain[10,12,13]. However, whether other components of young blood might also contribute to its anti-aging effects remain unknown.

One potential source of rejuvenating signals in plasma, which has not been tested to date, is exosomes. Exosomes are 40-150 nm vesicles which carry a wide array of proteins, mRNAs, and miRNAs[reviewed in 14,15,16], that can be delivered to recipient cells through fusion with the plasma membrane[17], phagocytosis[18], or via extracellular ligand-receptor signaling[19–21]. While a relatively newly identified form of intercellular communication, many studies have now

implicated exosomal signaling as a regulator of homeostatic and regenerative capacity in the brain, regulating synaptic plasticity and neurogenesis, *in vitro*[22]. Notably, recent studies suggest that exosomes may cross the blood-brain barrier[23–25], positioning them as a potential link between age-related changes in blood and brain aging. Moreover, young blood-derived exosomes have been shown to promote myelination in the aged rat motor cortex[26], further highlighting their potential for rejuvenation. We hypothesized that young plasma-derived exosomes confer the rejuvenating effects of young plasma administration on cellular, molecular, and behavioral hallmarks of hippocampal aging.

3.4 Results

Before assessing whether treatment with young plasma-derived exosomes rejuvenates hippocampal aging, we first sought to corroborate and extend the characterization of the rejuvenating effects of young plasma treatment on cellular, molecular, and cognitive hallmarks of hippocampal aging. To that end, plasma collected from young (3 month) mice was administered via intravenous injection to aged (17-18 month) recipients, 8 times over 24 days (**Figure 3.1a**). Hippocampal-dependent cognition was assessed in the contextual fear conditioning (FC) paradigm (**Figure 3.1b-c**). Consistent with previous reports[9], while no differences were observed in baseline freezing (**Figure 3.1b**), we found that aged mice treated with young plasma exhibited increased freezing in contextual FC compared to saline-treated controls (**Figure 3.1c**), indicating improved hippocampal-dependent memory. Additionally, we found that the effects of young plasma extend beyond those reported in hippocampus, as aged mice treated with young plasma exhibited increased freezing in the amygdala-dependent cued FC task (**Figure 3.1d**).

The cognitive enhancements elicited by young plasma treatment have previously been shown to depend on an increase in the phosphorylation of the cyclic AMP response element binding protein (CREB), a transcription factor that regulates synaptic strengthening[9,27]. In order

to corroborate and expand upon these findings, we assessed plasticity- and synaptic-marker expression in hippocampal lysate of aged (18 months) mice treated with plasma isolated from young (3 months) or aged (18 months) donors, by western blot analysis. Consistent with previous reports[9], treatment with young plasma increased levels of phosphorylated CREB (pCreb) in the hippocampi of aged mice (**Figure 3.1e-f**). Morever, we found that exposure to young plasma led to a significant increase in levels of the neurotrphic factor BDNF in aged hippocampi, and this increase was accompanied by concommittant increases in the levels of the presynaptic marker Synapsin-1, the post synaptic marker PSD95 (**Figure 3.1e-f**).

Another form of plasticity that declines with age in the hippocampus is neurogenesis[5,28]. While exposure to young blood has been shown to increase neurogenesis in the hippocampi of aged mice in the model of parabiosis[8], whether these benefits are transferred by young blood plasma remains to be elucidated. Therefore, we assessed neurogenesis in the hippocampi of aged mice treated with young plasma or saline, by immunohistochemical analysis of the number of doublecortin (DCX)-positive immature neurons. We observed a significant increase in the number of DCX+ cells in the hippocampi of aged mice treated with young plasma, compared to saline treated controls (**Figure 3.1g-h**).

In order to assess whether young plasma-derived exosomes confer benefits to the aged hippocampus, consistent with those observed after young plasma treatment, we first sought to validate our exosome isolation approach. Exosomes, were isolated from young mouse plasma by ultracentrifugation. Isolated exosomes exhibited a mode diameter of <150 nm, assessed by Nanosight Tracking Analysis (**Figure 3.2a**). The size and stereotypic donut-shaped morphology of plasma exosomes was confirmed by electron microscopy (**Figure 3.2b**), and exosomal enrichment was confirmed by the detection of the tetraspanins CD63 and CD9, by western blot analysis (**Figure 3.2c**).

In order to identify a physiological dose for exosome administration studies, we next sought to estimate the number of exosomes present per ml of mouse plasma. To that end,

exosomes were isolated from young mouse plasma, and were quantified with Nanosight Particle Tracking analysis. We collected 1.24*10^9 exosomes/ml plasma. We additionally assessed the efficiency of our isolation protocol, and found that we were able to re-isolate 22.5% +/- 3.4% (n=3; mean +/- SD) of exosomes from a known input. Therefore, we estimated that 1ml of mouse plasma contains 5.5*10^9 exosomes (1.24*10^9/22.5%), which is within the range of previous estimates of the number of exosomes present in plasma[29–31]. Accordingly, for rejuvenation studies we administered 5.5*10^8 exosomes in 100 ul PBS, to mimic the physiological number of exosomes estimated to be present in 100 ul of young mouse plasma, consistent with plasma administration studies[9] (**Figure 3.1a**).

In order to assess whether young plasma-derived exosomes contain rejuvenating capacity, exosomes isolated from young (2 month) mice were administered to aged (18 mo) recipients, by retro-orbital injections, 9x over 28 days, and hippocampal-dependent memory was assessed using contextual FC and radial arm water maze (RAWM) behavioral paradigms (**Figure 3.3a**). Exosome treated animals exhibited no difference in weight, compared to PBS treated controls (**Figure 3.3b**). In the FC paradigm, exosome treated aged mice exhibited a modest, but significant decrease in baseline freezing (**Figure 3.3c**). Despite this difference, treatment with young plasma-derived exosomes did not affect hippocampal- or amygdala-dependent memory in the contextual (**Figure 3.3d**) or cued (**Figure 3.3e**) FC paradigms compared to controls.

As an additional assessment of hippocampal-dependent learning and memory, aged mice treated with young plasma-derived exosomes or PBS were tested in the RAWM behavioral paradigm, which has been previously shown to be sensitive to aging and to the rejuvenating effects of young plasma administration[8,9]. No differences in learning or memory were observed in young plasma-derived exosome treated aged mice compared to controls, as assessed by the numbers of errors made during the training or testing portions of the task (**Figure 3.3f**). Moreover, no difference was observed in overall learning between exosome treated and control

mice (**Figure 3.3g**). Together these data indicate that young blood-derived exosomes do not improve hippocampal-dependent learning and memory.

We next examined whether exposure to young plasma-derived exosomes rejuvenated molecular or cellular hallmarks of hippocampal aging. We first assessed the effects of young plasma-derived exosome treatment on synaptic marker expression in the aged hippocampus, by western blot analysis. No differences were observed in hippocampal expression of the synaptic markers NR2B, NR2A, PSD95, or AMPAR (**Figure 3.3h-i**). At a cellular level, we assessed whether young plasma-derived exosomes promote hippocampal neurogenesis, and found that treatment with young-plasma derived exosomes did not affect the number of DCX+ cells in the hippocampi of aged mice (**Figure 3j-k**). These data suggest that administration of young plasma exosomes does not rejuvenate neurogenesis in the aged hippocampus.

Aging results in widespread impairments in the brain, and indeed, synaptic density has been shown to decline with age in the cortex, as well as the hippocampus[3]. However, whether exposure to young blood rejuvenates the aged cortex has not been studied. Therefore, we asked whether exposure to young plasma, or young plasma-derived exosomes, affect synaptic marker expression in the cortex of aged mice. We found that the rejuvenating effects of young plasma treatment were not confined to the hippocampus, as exposure to young plasma led to increased levels of pCREB, BDNF, Syn-1, and PSD-95 in cortical lysates from aged mice, compared to aged plasma treated controls (**Figure 3.4a-b**).

Notably, despite their lack of effect on the hippocampus, treatment with young plasma-derived exosomes led to increase expression of PSD95 in the cortex of aged mice (**Figure 3.5a-b**). Additionally, we observed a trend towards increased expression of the glutamate receptor subunit, NR2B, and no change in NR2A or AMPAR expression in the cortex of aged mice treated with young plasma-derived exosomes. Together this data suggests that young plasma-derived exosomes contain rejuvenating capacity, and may contribute to the rejuvenating effects of young plasma treatment on synaptic marker expression in the cortex.

3.5 Discussion

Cumulatively, our data indicate broad effects of young blood plasma treatment on molecular, cellular, and behavioral hallmarks of hippocampal aging. Indeed, we report for the first time that treatment with young plasma promotes neurogenesis and increases synaptic marker expression in the aged hippocampus. Moreover, we find that the beneficial effects of young blood plasma treatment extend beyond the hippocampus, as young plasma administration led to increased levels of synaptic marker expression in the cortex of aged mice. At a mechanistic level, we tested whether young plasma-derived exosomes contribute to the rejuvenating effects of young plasma treatment on hippocampal or cortical aging phenotypes. A number of studies have used nanosight particle tracking to determine the concentration of exosomes in human plasma, with estimates ranging 0.8*10^8 - 4.3*10^8 on the low end[29,30] to 9.25*10^9 vessicles/ml plasma on the high end[31]. We found that the concentration of exosomes in mouse plasma to be 5.5*10^9 exosomes/ml, consistent with these previous reports[29–31] and suggesting that we administered a physiologically relevant dose of exosomes. Despite this, we found that treatment with young plasma-derived exosomes did not recapitulate any of the benefits of young plasma administration on hippocampal aging. Interestingly, however, treatment with young plasma-derived exosomes did lead to increased synaptic marker expression in the cortex of aged mice, suggesting that they may play a role in rejuvenation outside of the hippocampus. Together, our findings indicate that the beneficial effects of young plasma treatment are differentially mediated, in a region specific manner. More work is now warranted to further investigate the role of young plasma and young plasma-derived exosome treatment on age-related impairments in cortex-dependent memory.

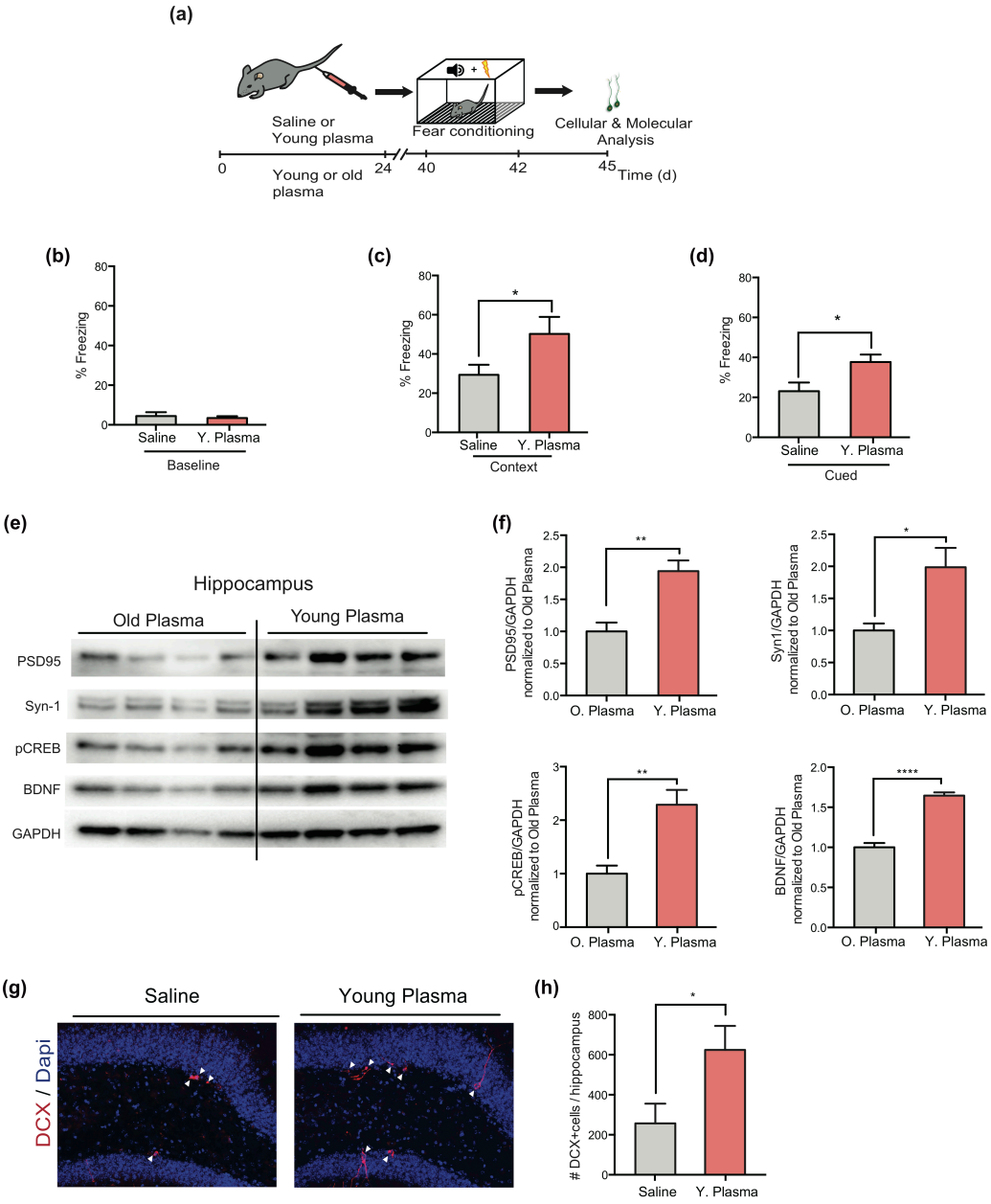


Figure 3.1 Young plasma administration rejuvenates the aged hippocampus. (**a**) Schematic showing young plasma administration paradigms into aged mice. (**b**) Assessment of baseline freezing in aged mice treated with young (y.) plasma or saline, during the training portion of the fear conditioning (FC) paradigm (n=6-8/group). (**c**) Hippocampal-dependent memory was assessed aged mice treated with young plasma or saline by contextual FC (n=6-8/group). (**d**) Amygdala-dependent memory was assessed in aged mice treated with young plasma or saline in the cued FC paradigm (n=6-8/group). (**e-f**) Representative western blot (**e**) and quantification (**f**) of hippocampal lysates from aged mice treated with young (y.) or old (o.) plasma, probed with anti-PSD95, Syn-1, pCREB, BDNF, and GAPDH (n=4/group). (**g-h**) Representative fields (**g**) and quantification (**h**) of DCX+ cells/hippocampus of aged mice treated with young plasma or saline (n=5-6/group). All data shown as the mean +*SEM*. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. t-test (**b-d,f,h**).

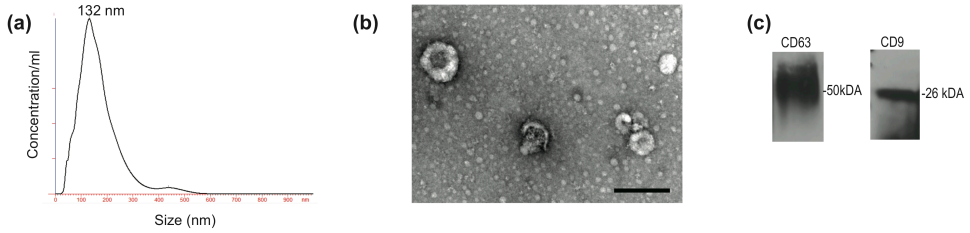


Figure 3.2 Isolation of exosomes from young mouse plasma. (**a**) Representative size distribution of young plasma-derived exosomes as determined by Nanosight Particle Tracking. (**b**) Representative electron microscopy image of young plasma-derive exosomes (scale bar = 200 nm). (**c**) Representative western blot of isolated exosomes probed for expression of the exosome markers CD63 and CD9.

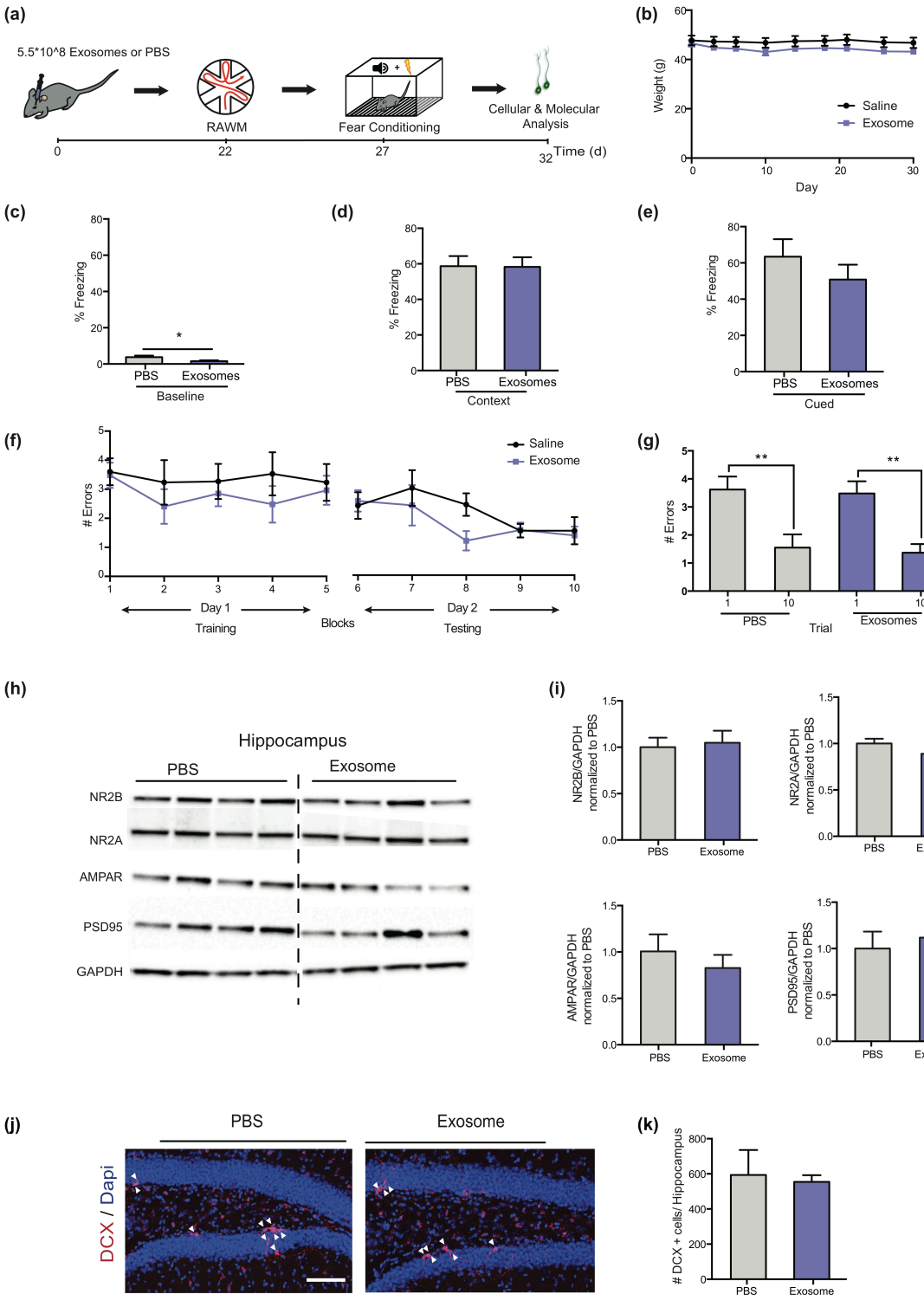

(a)
5.5*10^8 Exosomes or PBS
RAWM
Fear Conditioning
Cellular & Molecular Analysis
0
22
27
32
Time (d)
(b)
Weight (g)
Saline
Exosome
Day
(c)
% Freezing
PBS
Exosomes
Baseline
(d)
% Freezing
PBS
Exosomes
Context
(e)
% Freezing
PBS
Exosomes
Cued
(f)
Errors
Saline
Exosome
Day 1
Training
Blocks
Day 2
Testing
(g)
Errors
PBS
Trial
Exosomes
(h)
Hippocampus
PBS
Exosome
NR2B
NR2A
AMPAR
PSD95
GAPDH
(i)
NR2B/GAPDH normalized to PBS
NR2A/GAPDH normalized to PBS
AMPAR/GAPDH normalized to PBS
PSD95/GAPDH normalized to PBS
PBS
Exosome
(j)
PBS
Exosome
DCX / Dapi
(k)
DCX + cells/ Hippocampus
PBS
Exosome

Figure 3.3 Young plasma-derived exosomes do not rejuvenate the aged hippocampus. (**a**) Schematic showing young plasma-derived exosome administration paradigm in aged mice. (**b**) Weights of aged mice treated with young plasma-derived exosomes or PBS was assessed over the duration of the study (n=9-10/group). (**c**) Assessment of baseline freezing in aged mice treated with young plasma-derived exosomes or PBS, during the training portion of the fear conditioning (FC) paradigm (n=9-10/group). (**d**) Hippocampal-dependent memory was assessed in aged mice treated with young plasma-derived exosomes or PBS in the contextual FC paradigm (n=9-10/group). (**e**) Amygdala-dependent memory was assessed in aged mice treated with young plasma-derived exosomes or PBS in the cued FC paradigm (n=9/group). **(f-g**) Hippocampal-dependent memory was assessed aged mice treated with young plasma-derived exosomes or PBS in the radial arm water maze (RAWM) behavioral paradigm, by assessing the number of errors made before finding the hidden platform during the training and testing portions of the paradigm (**f**; n=9-10/group) or in the first and last trial of the RAWM paradigm (**g**; n=9-10/group). (**h-i**) Representative western blot (**h**) and quantification (**i**) of hippocampal lysates from aged mice treated with young plasma-derived exosomes or PBS, probed with anti-PSD95, NR2A, NR2B, AMPAR, and GAPDH (n=7-8/group). (**j-k**) Representative fields (**j**; scale bar = 100 um) and quantification (**k**) of DCX+ cells/hippocampus of aged mice treated with young plasma-derived exosomes or PBS (n=6-8/group). All data shown as the mean +*SEM*. **p<0.05*, ***p<0.01.* Repeated measures analysis of variance (ANOVA) with Sidak correction for multiple comparison (**b**,**f**). t-test (**c-e, g,i,k**).

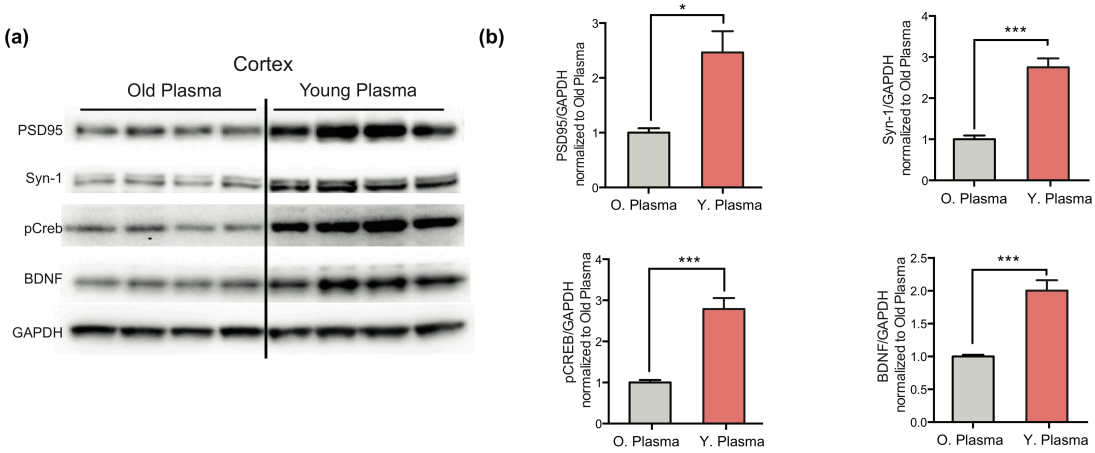


Figure 3.4. Young plasma administration increases expression of key proteins involved in synaptic function in the cortex of aged mice. (**a-b**) Representative western blot (**a**) and quantification (**b**) of cortical lysates from aged mice treated with young (Y.) or old (O.) plasma, probed with anti-PSD95, Syn-1, pCREB, BDNF, and GAPDH (n=4/group). All data shown as the mean +*SEM*. *$p<0.05$, **$p<0.01$, ***$p<0.001$. t-test (**b**).

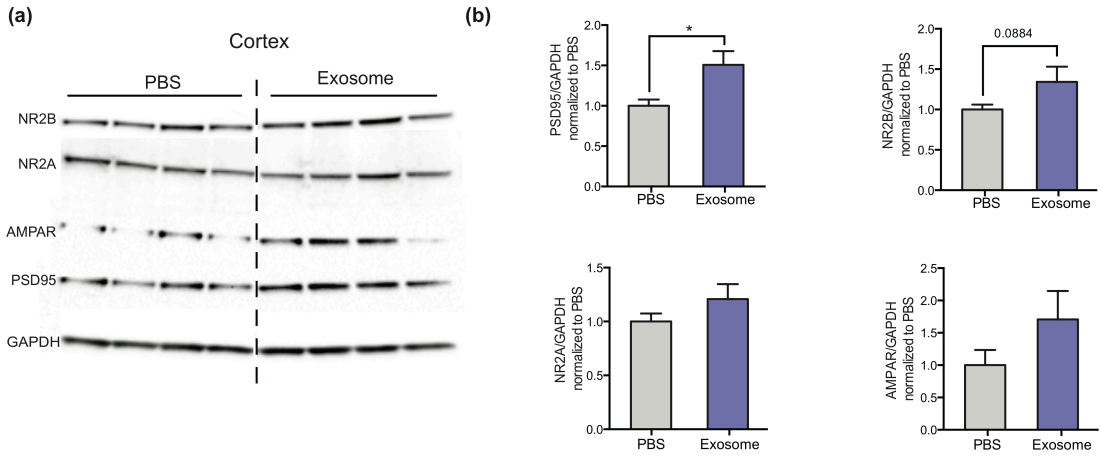


Figure 3.5. Young plasma-derived exosomes increase synaptic marker expression in the cortex of aged mice. (**a-b**) Representative western blot (**a**) and quantification (**b**) of cortical lysates from aged mice treated with young plasma-derived exosomes or PBS, probed with anti-NR2B, NR2A, AMPAR, PSD95, and GAPDH (n=7-8/group). All data shown as the mean +*SEM*. **p<0.05*, ***p<0.01.* t-test (**b**).

3.6 References

1. Mattson, M. P. & Magnus, T. Ageing and neuronal vulnerability. *Nat. Rev. Neurosci.* **7**, 278–94 (2006).
2. Hedden, T. & Gabrieli, J. D. E. Insights into the ageing mind: a view from cognitive neuroscience. *Nat. Rev. Neurosci.* **5**, 87–96 (2004).
3. Samson, R. D. & Barnes, C. A. Impact of aging brain circuits on cognition. *Eur. J. Neurosci.* (2013). doi:10.1111/ejn.12183
4. Leal, S. L. & Yassa, M. A. Neurocognitive Aging and the Hippocampus across Species. *Trends in Neurosciences* (2015). doi:10.1016/j.tins.2015.10.003
5. Gontier, G. *et al.* Tet2 Rescues Age-Related Regenerative Decline and Enhances Cognitive Function in the Adult Mouse Brain. *Cell Rep.* (2018). doi:10.1016/j.celrep.2018.02.001
6. Wheatley, E. G. *et al.* Neuronal O-GlcNAcylation Improves Cognitive Function in the Aged Mouse Brain. *Curr. Biol.* (2019). doi:10.1016/j.cub.2019.08.003
7. Oh, H., Lewis, D. A. & Sibille, E. The Role of BDNF in Age-Dependent Changes of Excitatory and Inhibitory Synaptic Markers in the Human Prefrontal Cortex. *Neuropsychopharmacology* (2016). doi:10.1038/npp.2016.126
8. Villeda, S. a *et al.* The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* **477**, 90–94 (2011).
9. Villeda, S. A. *et al.* Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat. Med.* **20**, 659–63 (2014).
10. Khrimian, L. *et al.* Gpr158 mediates osteocalcin's regulation of cognition. *J. Exp. Med.* (2017). doi:10.1084/jem.20171320
11. Castellano, J. M. *et al.* In vivo assessment of behavioral recovery and circulatory exchange in the peritoneal parabiosis model. *Sci. Rep.* **6**, 29015 (2016).
12. Castellano, J. M. *et al.* Human umbilical cord plasma proteins revitalize hippocampal

function in aged mice. *Nature* **544**, 488–492 (2017).

13. Katsimpardi, L. *et al.* Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science* **344**, 630–4 (2014).
14. EL Andaloussi, S., Mäger, I., Breakefield, X. O. & Wood, M. J. a. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* **12**, 347–57 (2013).
15. Vlassov, A. V., Magdaleno, S., Setterquist, R. & Conrad, R. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta - General Subjects* **1820**, 940–948 (2012).
16. Roma-Rodrigues, C., Fernandes, A. R. & Baptista, P. V. Exosome in tumour microenvironment: Overview of the crosstalk between normal and cancer cells. *BioMed Research International* **2014**, (2014).
17. Montecalvo, A. *et al.* Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* **119**, 756–766 (2012).
18. Feng, D. *et al.* Cellular internalization of exosomes occurs through phagocytosis. *Traffic* **11**, 675–687 (2010).
19. Gross, J. C., Chaudhary, V., Bartscherer, K. & Boutros, M. Active Wnt proteins are secreted on exosomes. *Nature Cell Biology* **14**, 1036–1045 (2012).
20. Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**, 1161–1172 (1996).
21. Blanchard, N. *et al.* TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J. Immunol.* **168**, 3235–3241 (2002).
22. Saeedi, S., Israel, S., Nagy, C. & Turecki, G. The emerging role of exosomes in mental disorders. *Translational Psychiatry* (2019). doi:10.1038/s41398-019-0459-9
23. Alvarez-Erviti, L. *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* (2011). doi:10.1038/nbt.1807

24. Chen, C. C. *et al.* Elucidation of Exosome Migration Across the Blood–Brain Barrier Model In Vitro. *Cell. Mol. Bioeng.* (2016). doi:10.1007/s12195-016-0458-3

25. Ridder, K. *et al.* Extracellular Vesicle-Mediated Transfer of Genetic Information between the Hematopoietic System and the Brain in Response to Inflammation. *PLoS Biol.* (2014). doi:10.1371/journal.pbio.1001874

26. Pusic, A. D. & Kraig, R. P. Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination. *Glia* (2014). doi:10.1002/glia.22606

27. Yu, X. W., Curlik, D. M., Oh, M. M., Yin, J. C. P. & Disterhoft, J. F. CREB overexpression in dorsal CA1 ameliorates long-term memory deficits in aged rats. *Elife* (2017). doi:10.7554/eLife.19358

28. Knoth, R. *et al.* Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PLoS One* (2010). doi:10.1371/journal.pone.0008809

29. Eman Abu-Seer. The Reliability of Plasma Exosome Concentrations in Healthy Male Individuals. *J. Heal. Sci.* (2017). doi:10.17265/2328-7136/2017.02.004

30. Huang, X. *et al.* Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics* (2013). doi:10.1186/1471-2164-14-319

31. Fernando, M. R., Jiang, C., Krzyzanowski, G. D. & Ryan, W. L. New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. *PLoS One* (2017). doi:10.1371/journal.pone.0183915

32. Théry, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. *Curr. Protoc. Cell Biol.* (2006). doi:10.1002/0471143030.cb0322s30

33. Smith, L. K. *et al.* β2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nat. Med.* **21**, 932–7 (2015).

34. Alamed, J., Wilcock, D. M., Diamond, D. M., Gordon, M. N. & Morgan, D. Two-day radial-

arm water maze learning and memory task; robust resolution of amyloid-related memory deficits in transgenic mice. *Nat. Protoc.* (2006). doi:10.1038/nprot.2006.275

3.7 Materials and methods

Mice. Young (2-3 months) and aged (17-18 mo) wild-type C57BL/6J mice were obtained from the Taconic Biosciences and subsequently maintained in-house under specific pathogen-free conditions under a 12-h light-dark cycle. All studies were done in male mice. The numbers of mice used to result in statistically significant differences was calculated using standard power calculations with $\alpha = 0.05$ and a power of 0.8. We used an online tool (http://www.stat.uiowa.edu/~rlenth/Power/index.html) to calculate power and sample size on the basis of experience with the respective tests, variability of the assays and inter-individual differences within groups. All animal handling and use was in accordance with institutional guidelines approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC).

Plasma isolation. Pooled mouse blood was collected with EDTA from young (2-3 month) or aged (18 month) mice by intracardiac bleed at time of euthanasia. Plasma was collected from EDTA blood by centrifugation at 1,000 xg, within 30 minutes of harvest. All plasma aliquots were stored at -80^{o}C until use.

Exosome Isolation. Exosomes were isolated from young (2 month) mouse plasma by differential ultracentrifugation, as previously described[32]. Briefly, plasma was diluted to 20 ml in ultrapure, 0.22 um filtered PBS, and spun down at 2,000g for 10 minutes to remove cells. The supernatant was then collected and spun at 10,000g for 30 minutes to remove cell debris and apoptotic bodies. The supernatant was then filtered through 0.22 um syringe filter, and exosomes were pelleted by ultracentrifugation at 100,000 g, for 90 minutes. Pelleted exosomes were then washed in 20 ml ultrapure PBS, and recollected at 100,000g for 90 minutes. Ultracentrifugation was done using SW 28 or Ti 70.1 rotors on a Beckmann Coulter Optima XPN-80 ultracentrifugation. The exosomes were then resuspended in ultrapure PBS for

downstream analysis by Nanosight Particle tracking, western blot, or electron microscopy, or for in vivo administration studies.

Nanoparticle tracking. The concentration and size distribution of the purified exosomes was determined using Nanisight nanoparticle tracking analysis (Malvern Inc.) To get consistent measurements on the nanoparticle tracking software, purified exosomes were diluted 5-fold or more in cold, filtered PBS to obtain an ideal vesicle concentration range. 3 measurements of each sample were collected and concentration values were averaged.

Transmission electron microscopy. Exosomes were fixed in EM grade formaldehyde immediately following isolation. Negative staining was performed on a small aliquot of the fixed exosomes, which were loaded on Formvar-carbon coated EM grids and negative staining was performed as previously described[32]. Scanning electron microscopy allowed visualization of vesicle structure and size.

Plasma administration. Before administration, plasma was dialyzed using 3.5-kDa D-tube dialyzers (EMD Millipore) in PBS to remove EDTA. Aged mice were systemically treated with plasma (100 ul per injection) isolated from young or aged mice intravenously into the tail vein eight times over 24 d.

Exosome administration. Exosomes, isolated from young mouse plasma, were administered to aged mice (to $5.5x10^9$ exosomes/ml in 100ul PBS), and systemically via intraorbital injection, 9 times over 28 days.

Western blot analysis. Isolated exosome preps were lysed in RIPA buffer (500 mM Tris, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, and complete protease

inhibitors; Roche). Lysates were stored at -80C or used immediately for protein analysis. Exosome lysates were mixed with 4x NuPage LDS loading buffer (Invitrogen) and loaded on a 10% SDS polyacrylamide gradient gel (Invitrogen) and subsequently transferred onto a nitrocellulose membrane. The blots were blocked in 5% milk in Tris-Buffered Saline with Tween (TBST) and incubated with rat anti-CD63 (1:500; MBL) and rat anti-CD9 (1:2000; Biosciences). Multiple exposures were taken to select images within the dynamic range of the film (GE Healthcare Amersham HyperfilmTM ECL). Selected films were scanned (300 dpi) and quantified using ImageJ software (Version 1.52)

For analysis of synaptic markers in the brain, the hippocampus or cortex were dissected after perfusion of animals, snap frozen, and lysed in RIPA lysis buffer (500 nM Tris, pH7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and complete protease inhibitors; Roche). Samples were mixed with 4x NuPage LDS loading buffer (Invitrogen) and loaded on a 4-12% SDS polyacrylamide gradient gel (Invitrogen) and subsequently transferred onto a nitrocellulose membrane. The blots were blocked in 5% milk in TBST and incubated with rabbit anti-pCREB (1:1000; Cell Signaling), rabbit anti-AMPAR (1:3000; Abcam), mouse anti-Synaptophysin (1:1000; Millipore), rabbit anti-NR2B (1:2000; Millipore), rabbit anti-NR2A (1:4000; Millipore), rabbit anti-synapsin-1 (1:5000; Abcam), rabbit anti-BDNF (1:500; invitrogen), and mouse anti-GAPDH (1:5000; Abcam). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) kit (GE Healthcare) were used to detect protein signals. Membranes were imaged using a Chemidoc system (BioRad) or exposed to film (GE Healthcare Amersham HyperfilmTM ECL) and scanned (300 dpi). Images were quantified using ImageJ software (Version 1.52a). GAPDH bands were used for normalization for brain lysates.

Immunohistochemistry. Tissue processing and immunohistochemistry was performed on free-floating sections according to standard published techniques[33]. Mice were anesthetized with

87.5 mg/kg ketamine and 12.5mg/kg xylazine and transcardially perfused with 1x phosphate buffered saline (PBS). Brains were removed and fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4^{o}C for 48 h before cryoprotection in 30% sucrose. Brains were sectioned coronally, at 40 uM with a cryomicrotome (Leica Camera, Inc.) and stored in cryoprotective medium. Primary antibodies used were: goat anti-DCX (1:7000; Santa Cruz). After overnight incubation, unconjugated primary antibody staining was revealed using fluorescence-conjugated secondary antibodies (Life Technologies). All cells were counted in the DG of every sixth coronal hemibrain section through the hippocampus and analyzed by confocal or epifluourescent microscopy using a Zeiss Axio Observer.Z1 or Axio Imager M2 microscope, respectively.

Radial arm water maze. Spatial learning and memory was assessed using the radial arm water maze (RAWM) paradigm according to established protocol[34]. In this task the goal arm location containing a platform remains constant throughout the training and testing phase, while the start arm is changed during each trial. On day one during the training phase, mice are trained for 15 trails, with trials alternating between a visible and hidden platform. On day two during the testing phase, mice are tested for 15 trials with a hidden platform. Entry into an incorrect arm is scored as an error, and errors are averaged over training blocks (three consecutive trials).

Contextual fear conditioning. In this task, mice learned to associate the environmental context (fear conditioning chamber) with an aversive stimulus (mild foot shock; unconditioned stimulus, US) enabling testing for hippocampal-dependent contextual fear conditioning. As contextual fear conditioning is hippocampus and amygdala dependent, the mild foot shock was paired with a light and tone cue (conditioned stimulus, CS) in order to also assess amygdala-dependent cued fear conditioning. Conditioned fear was displayed as freezing

behavior. Specific training parameters are as follows: tone duration is 30 s; level is 70 dB, 2 kHz; shock duration is 2 s; intensity is 0.6 mA. This intensity is not painful and can easily be tolerated but will generate an unpleasant feeling. On day 1 each mouse was placed in a fear-conditioning chamber and allowed to explore for 2 min before delivery of a 30-s tone (70 dB) ending with a 2-s foot shock (0.6 mA). Two minutes later, a second CS-US pair was delivered. On day 2, each mouse was first placed in the fear-conditioning chamber containing the same exact context, but with no CS or foot shock. Freezing was analyzed for 2 min. One hour later, the mice were placed in a new context containing a different odor, cleaning solution, floor texture, chamber walls and shape. Animals were allowed to explore for 2 min before being re-exposed to the CS. Freezing was analyzed for 30s after re-exposure to CS. Freezing was measured using a FreezeScan video tracking system and software (Cleversys, Inc).

Data and statistical analysis. Mice were randomized prior to treatment. Researchers were blinded throughout histological, biochemical and behavioral assessments. Groups were un-blinded at the end of each experiment upon statistical analysis. Data are expressed as mean +*SEM*. The distribution of data in each set of experiments was tested for normality using D'Agostino-Pearson omnibus test or Shapiro-Wilk test. No significant differences in variance between groups were detected using an F test. Statistical analysis was performed with Prism 6.0 software (GraphPad Software). Means between two groups were compared with two-tailed, unpaired Student's *t*-test. Comparisons of means from multiple groups with each other or against one control group were analyzed with one-way ANOVA followed by appropriate *post hoc* tests (indicated in figure legends). The data that support the findings of this study are available from the corresponding author upon reasonable request.

Publishing Agreement

It is the policy of the University to encourage open access and broad distribution of all theses, dissertations, and manuscripts. The Graduate Division will facilitate the distribution of UCSF theses, dissertations, and manuscripts to the UCSF Library for open access and distribution. UCSF will make such theses, dissertations, and manuscripts accessible to the public and will take reasonable steps to preserve these works in perpetuity.

I hereby grant the non-exclusive, perpetual right to The Regents of the University of California to reproduce, publicly display, distribute, preserve, and publish copies of my thesis, dissertation, or manuscript in any form or media, now existing or later derived, including access online for teaching, research, and public service purposes.

DocuSigned by:
Lucas Smith
10A534AE9830492...
Author Signature

6/25/2020
Date