Loss of Caveolin-1 Accelerates Neurodegeneration and Aging

Brian P. Head1,2,*, Jason N. Peart4, Mathivadhani Panneerselvam1, Takaakira Yokoyama1, Matthew L. Pearn1, Ingrid R. Niesman1, Jacqueline A. Bonds3, Jan M. Schilling1,2, Atsushi Miyahara5, John Headrick4, Sameh S. Ali3, David M. Roth1,2, Piyush M. Patel1,2,*, Hemal H. Patel1,2,9

1 Department of Anesthesiology, University of California San Diego, La Jolla, California, United States of America, 2 VA San Diego Healthcare System, San Diego, California, United States of America, 3 Department of Medicine, University of California, La Jolla, California, United States of America, 4 Heart Foundation Research Centre, Griffith University, Gold Coast, Queensland, Australia, 5 Gene Therapy Program, University of California San Diego, La Jolla, California, United States of America

Abstract

Background: The aged brain exhibits a loss in gray matter and a decrease in spines and synaptic densities that may represent a sequela for neurodegenerative diseases such as Alzheimer’s. Membrane/lipid rafts (MLR), discrete regions of the plasmalemma enriched in cholesterol, glycosphingolipids, and sphingomyelin, are essential for the development and stabilization of synapses. Caveolin-1 (Cav-1), a cholesterol binding protein organizes synaptic signaling components within MLR. It is unknown whether loss of synapses is dependent on an age-related loss of Cav-1 expression and whether this has implications for neurodegenerative diseases such as Alzheimer’s disease.

Methodology/Principal Findings: We analyzed brains from young (Yg, 3-6 months), middle age (Md, 12 months), aged (Ag, >18 months), and young Cav-1 KO mice and show that localization of PSD-95, NR2A, NR2B, TrkB, AMPAR, and Cav-1 to MLR is decreased in aged hippocampi. Young Cav-1 KO mice showed signs of premature neuronal aging and degeneration. Hippocampal synaptosomes from Cav-1 KO mice showed reduced PSD-95, NR2A, NR2B, and Cav-1, an inability to be protected against cerebral ischemia-reperfusion injury compared to young WT mice, increased Aβ, P-Tau, and astrogliosis, decreased cerebrovascular volume compared to young WT mice. As with aged hippocampi, Cav-1 KO brains showed significantly reduced synapses. Neuron-targeted re-expression of Cav-1 in Cav-1 KO neurons in vitro decreased Aβ expression.

Conclusions: Therefore, Cav-1 represents a novel control point for healthy neuronal aging and loss of Cav-1 represents a non-mutational model for Alzheimer’s disease.

Introduction

Cognitive decline is emerging as one of the greatest health problems in the elderly population [1,2]. Age alone increases the risk of stroke, Alzheimer’s disease (AD), and other forms of dementia [2]. The risk of AD increases 14-fold between the ages of 65–85, and affects almost 47% over the age of 85 [3].

Multiple signaling pathways regulate neuronal survival and growth to facilitate the formation of synapses and this signaling is altered with age [4,5,6,7]. Synapses are essential for learning, memory and the development of neurons in the CNS [8]. Receptors and associated proteins aggregate to mold and shape post-synaptic densities in order to permit high fidelity signal transduction leading to rapid regulation of neuronal function [9,10,11]. Understanding the basic pathophysiological mechanisms of cognitive decline and how the subcellular organization of signaling molecules is altered with cognitive decline could potentially yield novel therapeutic targets for neuronal aging and neurodegeneration.

Cholesterol is a major lipid component of synapses and a limiting factor in synapse development, synaptic activity, and neurotransmitter release [12]. Age-related impairments in the biosynthesis, transport, or uptake of cholesterol by neurons in the CNS may adversely affect development, plasticity, and synaptic circuitry associated with neurodegenerative diseases [13,14,15,16,17]. Membrane lipid rafts (MLR), discrete regions of the plasma membrane enriched in cholesterol, glycosphingolipids and sphingomyelin, are essential for synapse development, stabilization, and maintenance [12,18]. Moreover, caveolin-1 (Cav-1), a cholesterol binding and resident protein of MLR [19,20,21], organizes and targets synaptic components of the neurotransmitter and neurotrophic receptor signaling pathways.
to MLR [e.g., NMDAR, AMPAR, TrkR, Src Family Kinases (SFK)] [22,23,24,25,26,27]. Additionally, neurotransmitter and neurotrophic receptors are found within MLR in growth cones, a finding that has major implications for neuronal plasticity [11,20].

Early-onset AD, which afflicts individuals prior to 60–65 years of age, is known to be caused by mutations in three genes: amyloid precursor protein (APP), presenilin-1, and presenilin-2 [29]. MLR and cholesterol play a protective role against APP processing and amyloid-β (Aβ) toxicity [13,14,16,30,31,32,33]. Cav-1 KO mice develop CNS pathology similar to AD, such as altered NMDA receptor signaling, motor and behavioral abnormalities, increased ischemic cerebral injury, impaired spatial memory, and cholinergic function [27,34,35,36]. Whether MLR, Cav-1 expression, and the organization of pro-survival and pro-growth signaling mechanisms are altered in neurodegenerative states (age-related dementia and AD) has yet to be investigated. The present study tested whether 1) Cav-1 organizes synaptic signaling components in neuronal MLR and synaptosomes, 2) the localization of synaptic signaling components to neuronal MLR and synaptosomes is reduced in brains from aged wild-type and young Cav-1 KO mice, and 3) brains from Cav-1 KO mice develop a neuropathological phenotype similar to Alzheimer’s disease.

Results

PSD-95, NR2A, NR2B, and Cav-1 protein expression is decreased in middle aged and aged hippocampus

Hippocampi were isolated from brains of C57BL/6J mice (wild-type, WT) at 3–6 months (young), 12 months (middle aged), and >18 months (aged). Immunoblots of hippocampal homogenates showed a significant reduction in PSD-95 (n = 6, p = 0.0001 vs Md, p = 0.01 vs Ag), NR2A (n = 6, p = 0.02 vs Md, p = 0.02 vs Ag), NR2B (n = 6, p = 0.02 vs Md, p = 0.04 vs Ag), TrkB (n = 6, p = 0.009 vs Md, p = 0.03 vs Ag), and Cav-1 (n = 6, p = 0.008 vs Md, p = 0.04 vs Ag) in hippocampi from middle aged and aged mice when compared to young mice (Figure 1). These data demonstrate an age-dependent reduction in synaptic signaling components and Cav-1 in the hippocampus.

Age-related decreases in synaptic signaling components from MLR

MLR play a role in stabilizing synapses in the mammalian brain [12,18], therefore we performed sucrose density fractionation of whole brain homogenates from young, middle aged and aged WT mice to purify MLR. Immunoblots showed buoyant fractions from young brains contained the majority of PSD-95 (n = 5, p = 0.03 vs Ag), NR2A (n = 5, p = 0.04 vs Md, 0 = 0.0005 vs Ag), NR2B

Figure 1. Hippocampal homogenates show an aged dependent reduction in NR2A, NR2B, PSD-95, and Cav-1. Hippocampi were isolated from the brains of C57BL/6J mice at 3–6 months (young, Yg), 12 months (middle aged, Md), and 24 months (aged, Ag). Immunoblot and densitometric analysis demonstrated a significant reduction in PSD-95, NR2A, NR2B, TrkB, and Cav-1 in the Md and Ag hippocampus compared to Yg. doi:10.1371/journal.pone.0015697.g001
Young Cav-1 KO mice demonstrate accelerated aging and neurodegeneration

Loss of synaptic proteins and neuronal preconditioning. Cav-1 expression is decreased in hippocampi and buoyant fractions (i.e., MLR) from Ag mice (Figure 1 and 2), and we therefore assessed whether Yg Cav-1 KO mice displayed reduced synaptic protein expression. Hippocampal synaptosomes from Yg Cav-1 KO mice showed a similar pattern to Ag WT mice, reduced protein expression of PSD-95, NR2A, NR2B, and AMPAR (Figure 3A). Similar to aged WT mice, PSD-95 immunoprecipitations of hippocampal synaptosomes from Cav-1 KO mice showed minimal detection of PSD-95, NR2A, NR2B, and AMPAR.

We next sought to determine whether neuroprotection against ischemic injury is absent in Yg Cav-1 KO mice. To achieve this we performed an ischemic preconditioning protocol. Ischemic preconditioning (IPC), a phenomenon wherein sublethal ischemia protects the brain from a subsequent lethal ischemic event, is absent in brains from aged animals [38,39] and in neurons in vitro that have reduced or no Cav-1 expression [27]. We show here for the first time that Cav-1 KO mice show a similar reduction in neuroprotective signaling components to that exhibited by brains from aged WT mice. IPC significantly protected CA1 neurons against lethal ischemia in WT mice (n = 7, p = 0.0072 vs Li) (Figure 3B-iv, C). There was no significant protection in CA1 neurons from Cav-1 KO mice subjected to IPC prior to Li (Figure 3B-viii, C), demonstrating an inability to induce IPC in these mice. In terms of expression and function of synaptic signaling components, young Cav-1 KO mice resemble aged WT mice.

Early on-set of AD-like phenotype

Previous work has shown that Cav-1 and MLR can regulate amyloidogenic processing of APP [30]. Therefore, we assessed whether brains from Cav-1 KO mice exhibit pathological signs indicative of AD. Amyloid-β (Aβ) (n = 4, p = 0.005) and P-Tau[Thr181] (n = 4, p = 0.02) were significantly elevated in hippocampal homogenates from Yg Cav-1 KO mice (Figure 4A). Immunofluorescence microscopy demonstrated that Yg Cav-1 KO mice had increased Ab staining in Nissl positive neurons in the CA3 (n = 3, p = 0.006) and CA1 (n = 3, p = 0.04) region of the hippocampus compared to WT mice (Figure 4B). hippocampi from Cav-1 KO mice showed a 20–25% reduction in cerebrovascular volume (n = 4, p = 0.001) (GSA, blood vessel marker - Figure 4C).

Toluidine blue staining of the hippocampus showed a large reduction in neurons within the dentate gyrus and CA1 regions of Yg Cav-1 KO mice (Figure 5A-i, A-ii) compared to Yg (Figure 5C-iii, C-iv) and Ag (Figure 5B-i, B-ii) WT mice. In addition, there appeared to be more gli and glial scar formation within the dentate gyrus of Cav-1 KO mice as indicated by the darker gray cell bodies intermixed with the neurons (Figure 5A-iii, A-iv). Young Cav-1 KO show increased astrogliosis (n = 4, p = 0.0006) (GFAP, astrocyte marker – Figure 5D). Flours-Jade B staining demonstrated little neuronal degeneration and well-organized astrocytes in the CA1 from Yg WT mice when compared with Yg Cav-1 KO mice, which showed disorganized astrocytes and areas of potential plaque development. Due to their shorter life span [40], obtaining Ag Cav-1 KO mice is difficult. We here show that the CA1 region from 12 month Cav-1 KO mice had large bright, entangled green fluorescence with red fluorescent (Nissl) neurons and severely less organized astrocytes, demonstrating increased neuronal degeneration (Figure 5E).

There is a reduction in synaptic proteins from hippocampal synaptosomal membranes, which we therefore assessed whether Cav-1 KO mice exhibit changes in total hippocampal synapses. Routine electron microscopy (EM) revealed a significant reduction in hippocampal synapses (i.e., post synaptic densities) in both Cav-1 KO (n = 6, p = 0.002) (Figure 6C) and Ag (n = 6, p = 0.02) (Figure 6B) mice compared to Yg (Figure 6A). In addition, Cav-1 KO mice displayed unorganized cytoskeletal assemblage (arrow heads) within dendrites (d, asterisks) (Figure 6F) and elevated astrocyte presence (arrows) compared to brains from Ag (Figure 6E) and Yg WT mice (Figure 6D), the latter displaying normal cytoskeletal organization (arrow heads) within dendrites (d). These data indicate that Cav-1 KO mice develop pathological changes at 3 months of age consistent with aging and AD mouse models.

Re-expression of Cav-1 in Cav-1 KO neurons decreases Aβ

Cav-1 KO mice demonstrate pathology similar to AD such as elevated Aβ production in the hippocampus. We tested whether neuron-targeted re-expression of Cav-1 in primary Cav-1 KO neurons would decrease Aβ expression. We generated a viral vector that contains a neuron-specific synapsin promoter upstream of Cav-1 cDNA (SynCav1) (Figure 7A). Increasing doses of SynCav1 for 72 hr proportionally increased Cav-1 expression and reduced Aβ (Figure 7B). Six separate neuronal cultures from Cav-1 KO mouse brains were transfected with SynGFP (control vector) or SynCav1, and SynCav1 significantly reduced Aβ expression (n = 6, p = 0.002) after 72 hr (Figure 7C).

Discussion

The present study is the first to demonstrate that the cholesterol binding and MLR resident protein, Cav-1, complexes with synaptic proteins in the CNS, and that this organization is disrupted with age. Furthermore, this study is the first to demonstrate that loss of Cav-1 in a transgenic mouse model produces neuropathology similar to that exhibited with AD, i.e., Aβ production, elevated astrogliosis, reduced cerebrovasculature
Decreased Caveolin-1 and Neurodegeneration

**A**
- Yg
  - PSD-95
  - NR2A
  - NR2B
  - AMPAR
  - TrkB
  - Cav-1
- Md
  - PSD-95
  - NR2A
  - NR2B
  - AMPAR
  - TrkB
  - Cav-1
- Ag
  - PSD-95
  - NR2A
  - NR2B
  - AMPAR
  - TrkB
  - Cav-1

**B**
- PSD-95
  - Yg
  - Md
  - Ag
  - BF
  - HF
- NMDAR2A
  - Yg
  - Md
  - Ag
  - BF
  - HF

**C**
- Immunoprecipitation of BF
  - Yg
  - Md
  - Ag
  - C
  - P
  - PSD-95
  - NR2A
  - NR2B
  - AMPAR
  - TrkB
  - Cav-1

**D**
- Hpc Synaptosomes
  - Yg
  - Md
  - Ag
  - PSD-95 IPs
  - Yg
  - Md
  - Ag

**E**
- 5-DSA
  - Membrane order parameter
  - Young
  - Old
  - T₁
  - T₂
  - Membrane order parameter
  - *
and neuronal loss in the hippocampus. Our data suggest that not only are MLR and Cav-1 essential for maintaining and stabilizing proper synaptic signaling [27] and neuroprotection against cerebral ischemia, but they also may serve to alter amyloidogenic process of APP seen in AD brains. Lastly, Cav-1 KO mice may serve as the first non-mutational model of AD.

It is essential to understand the basic neural mechanisms of synapse formation and stabilization in order to identify potential therapeutic targets for facilitating neuronal regeneration and recovery of neuronal networks in the aged and injured brain. Traditionally synapses and MLR are considered separate subcellular structures, yet they both contain identical physical characteristics that are essential such as cholesterol, glycosphin-golipids, sphingomyelin, and other saturated fatty acid containing lipids (GM1 gangliosides, palmitic acid) as well as signaling components [22,23,24,25,26,27]. Growing evidence supports the role for free cholesterol and MLR in neuronal synaptic formation, signaling and protection [12,18,27,41,42,43]. Because free cholesterol directly affects Cav-1 expression, factors that alter intracellular cholesterol also change Cav-1 expression [44,45,46]. Specifically, brain derived neurotrophic factor (BDNF), a neurotrophin essential to synaptic function and development [47] which facilitates of long-term potentiation[48,49], elicits cholesterol biosynthesis and increased MLR and Cav expression in cortical and hippocampal neurons.[50] Furthermore, MLR are critical for growth cone expansion, neurite outgrowth, and axonal branching and guidance [11,51,52]. Therapeutic approaches to promote axonal reorganization and synapse formation after spinal cord injury use a MLR marker, cholera toxin B, as a direct indicator of axonal regeneration and de novo synapse formation [53,54]. Moreover, there exists increasing evidence that disruption or alterations of neuronal MLR and intracellular cholesterol can be neurotoxic and even contribute to enhanced neuronal vulnerability to Ab [13,14,33], demonstrating the importance of these distinct microdomains for proper pro-survival neuronal signaling [27,41,55,56,57]. When Cav-1 was over-expressed in β-secretase expressing cells, amyloid precursor protein and β-secretase localization to MLR resulted in decreased Ab production, suggesting a protective role by Cav-1 and MLR against Ab toxicity [30,31,32,58]. Interestingly the fatty acid content in MLR (a.k.a. detergent-resistant membranes, or DRMs) isolated from synaptic endings is altered in aged animals [59]. This result is consistent with our findings that membrane fluidity in synaptosomal membranes is increased in aged brains. Age-related physiochemical changes to distinct biological membranes such as MLR could be responsible for changes in Cav-1 expression and loss of synaptosomal pro-survival signaling components with age.

Our results demonstrate that loss of Cav-1 results in accelerated aging. Cav-1 KO mice have a shortened life span [40]. Two pathophysiologies altered with aging are vulnerability to ischemic stress and progression of AD. IPC is a phenomenon whereby brief ischemia, which does not injure neurons, renders the brain less vulnerable to subsequent ischemic injury [27,60,61,62,63,64]. IPC activates endogenous signaling pathways that are neuroprotective, and this neuroprotection is lost in the aged brain [38,39]. The underlying mechanism for the lack of ischemic tolerance in the aged brain is not clear. Signaling pathways in neurons are severely compromised with age. Specifically, post-synaptic molecules such as glutamate receptors, neurotrophin receptors and pro-survival signaling cascades (i.e., kinase activation and cAMP production) decrease significantly with age [65,66,67,68,69]. It is therefore possible that the organization, and thus efficacy of signaling pathways that produce tolerance is severely limited in the aged brain. We show in young Cav-1 KO mice that preconditioning is absent, suggesting a link between the loss of MLR and disrupted organization of pro-survival signaling.

In addition to loss of IPC, Cav-1 KO mice also exhibit characteristics consistent with AD. Cerebrovascular changes and increased astrogliaisis [70,71,72,73,74] coule also be a contributing factor to the absence of ischemic tolerance [75] as well as the AD phenotype exhibited by young Cav-1 KO mice. Upregulation of endogenous protective signaling in aged neurons through neuron-targeted Cav-1 expression might reduce the vulnerability of the aged brain even in the presence of reduced cerebrovascular volume. Neuron-targeted Cav-1 re-expression/over-expression offers the novel possibility of re-establishing the fidelity of neuroprotective signaling that is lost with advanced age or in other forms of neurodegeneration (i.e., dementia, Alzheimer's disease, depression, Parkinson's disease).

In summary, these findings demonstrate an important role for Cav-1 and MLR in organizing synaptic pro-survival signaling components that are essential for neuroprotection against ischemic injury, neuronal regeneration, and maintaining synapse stabilization and formation. Cav-1 may be a control point for neurological aging. Further understanding of how MLR and Cav-1 serve as a nexus for pro-survival and pro-growth signaling components may not only provide potential therapeutic targets for the preservation of neuronal function, but may also yield tools that could augment the brain's capacity to reorganize its neuronal networks following injury or during late stages of neurodegenerative diseases such as AD and other forms of dementia.

Materials and Methods

All studies performed on animals were approved by Veteran Affairs San Diego Institutional Animal Care and Use Committee (Protocol#: 08-035 and ID#:1141788) and conform to relevant National Institutes of Health guidelines.
Figure 3. Ischemic preconditioning (IPC) does not occur in Cav-1 KO mice. (A) Hippocampal synaptosomes from Cav-1 KO (Yg) showed a similar pattern to Ag, with a decrease in PSD-95, NR2A, NR2B, and AMPAR. PSD-95 IPs of Cav-1 KO synaptosomes revealed minimal detection in PSD-95, NR2A, NR2B, and AMPAR. (B) WT or Cav-1 KO mice were subjected to 3 min (ischemic preconditioning, IPC) and/or 12 min (lethal ischemia, LI) induced by bilateral carotid artery occlusion (BCAO). Intact neurons in CA1 hippocampal (HP) region were counted from Cresyl Violet stained paraffin fixed sections. IPC (3 min, BCAO) significantly protected CA1 neurons against LI (12 min, BCAO) in WT mice (iv). There was a significant increase in CA1 neuronal death in Cav-1 KO animals subject to IPC (viii) versus WT IPC + LI. Representative Cresyl Violet stained CA1 hippocampal images from (i) WT sham, (ii) WT IPC, (iii) WT LI, and (iv) WT IPC and (v) Cav-1 KO sham, (vi) Cav-1 KO IPC, (vii) Cav-1 KO LI, and (viii) Cav-1 KO IPC. Quantitation of images is presented by the graph.

doi:10.1371/journal.pone.0015697.g003
Primary neuron isolation and culture

Neonatal mouse neurons were isolated using a papan dissociation kit (Worthington Biochemical, Lakewood, NJ), as previously described [27]. Neurons were cultured in Neurobasal A media supplemented with B27 (2%), 250 mM GLUTMax1, P/S (1%). Cells were cultured on poly-D-lysine/laminin (2 µg/cm²) coated plates at 37 °C in 5% CO₂ for 4 d prior to transfection with lentiviral vectors. Cav-1 cDNA was cloned in our laboratory and given to Dr. Atushi Miyanohara at the UCSD Viral Vector Core. Dr. Miyanohara successfully generated a lentiviral vector containing the synapsin promoter up-stream of the Cav-1 gene (SynCav1). SynGFP was used as control vector. Titer for both vectors was approximately 10⁷ infectious units (i.u.) per ml.

Sucrose-density fractionation

Membrane/lipid rafts were isolated from adult brain and neurons using detergent-free methods. Tissues and cells were homogenized in sodium carbonate (150 mM, pH 11.0), and then sonicated with three cycles of 20 sec bursts with 1 min incubation on ice. Homogenate (1 mL) was mixed with 1 mL of 80% sucrose to generate 2 mL of 40% sucrose. Above the 40% layer, 6 mL of 1.2 M sucrose (41 g/100 ml or 41% sucrose) was carefully layered. The mixture was centrifuged at 175,000 g using SW41Ti rotor (Beckman) for 35% and 4 mL of 5% sucrose were carefully layered. The mixture was centrifuged at 175,000 g using SW41Ti rotor (Beckman) for 3 h at 4 °C. Samples were removed in 1 ml aliquots and the membrane/lipid rafts are found in buoyant fractions 4–5 (5/35% interface).

Synaptosomal membrane preparation

Neuronal cells or brain tissue were homogenized in 5 ml of solution A [0.32 M sucrose (34 g/500 ml), 0.5 mM CaCl₂ (36 mg/500 ml), 1 mM NaHCO₃ (42 mg/500 ml), 1 mM MgCl₂ (102 mg/500 ml)] containing protease and phosphatase inhibitors and used for immunoprecipitation [78]. The fluidity parameters were defined in [76,77]. The number designation indicates the relative position of the nitroxide on the stearic acid relative to the polar carboxylic group. In the case of 5-DSA, the spin probe is firmly held in place by the head groups of the lipids, which is reflected in broad EPR lines. Synaptosomes from young (3–6 m) and aged (>18 m) mice were isolated as described previously [78].

In vivo BCAO (bilateral carotid artery occlusion) model of neuronal preconditioning

Male C57BL/6J and Cav-1 KO mice were anesthetized with isoflurane. After endotracheal intubation, the lungs were mechanically ventilated with 1.5% isoflurane in 30% O₂, balanced N₂. Pericranial temperature was controlled at 37 °C. Via a pre-tracheal incision, the carotid arteries and the basilar artery were exposed and a temporary clip was applied to the basilar artery. Thereafter, preconditioning (PC) was induced by occlusion of the carotid arteries. The clips were removed after a defined interval (3 min for PC and 10 min for lethal ischemia), the wounds were infiltrated with 0.25% bupivacaine and the anesthetic was discontinued. Upon resumption of spontaneous ventilation, the endotracheal tube was removed and the animals were transferred to the animal care facility 4 h post extubation. Animals underwent transcerebral perfusion with heparinized saline followed by buffered paraformaldehyde. The brains were removed and the extent of injury to the CA1 sector of the hippocampus was determined by Cresyl violet staining.

Routine and immunoelectron microscopy

Brains were transcardially perfused fixed with standard Karnovsky’s fix, 4% paraformaldehyde, 1% glutaraldehyde, 0.1 M cacodylate buffer with 5 mM CaCl₂. NFD5-7 animals were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer and 5 mM CaCl₂ to prevent tissue artifacts. Hippocampi were dissected from whole brains after 24 h and 400 µm vibratome slices prepared and re-fixed an additional 24 h. Brains were blocked (i.e., dissected) to include hippocampal areas, one hemisphere for sagittal orientation, and one hemisphere for coronal. Blocks were re-fixed for an additional 24 h followed by post-fixation with 1% OsO₄ in 0.1 M cacodylate buffer, en bloc stained with uranyl acetate and embedded with flat orientation to locate appropriate hippocampal regions of interest. Each block was thick sectioned, stained with toluidine blue, and re-trimmed to

Figure 4. Aβ, A4 protein, and P-Tau(T181) are elevated in the hippocampus of young Cav-1 KO mice. (A) Hippocampal homogenates from WT (3 m) and Cav-1 KO (Cav-1 KO, 3–6 m) C57BL/6J mice were immunoblotted for Aβ and phosphorylated Tau (P-Tau[T181]), and GAPDH. Aβ and P-Tau(T181) were significantly elevated in young Cav-1 KO hippocampal homogenates. (B) Immunofluorescence microscopy showed that Cav-1 KO CA1 region of the hippocampus displayed elevated Aβ staining (green) overlapping with Nissl positive neurons (red) as indicated by yellow fluorescence. Quantitation of the data is represented in the graph. (C) Cryostat sections (50 µm) of mouse hippocampus were stained with lectin GSA (Griffonia simplicifolia) to label blood vessels. There was a 20–25% reduction in overall area occupied by blood vessels in Cav-1 KO. Quantitation of the data is represented in the graph (right). doi:10.1371/journal.pone.0015697.g004

Determination of synaptosomal membrane fluidity using electron paramagnetic resonance (EPR)

Hydrocarbon chain mobility was measured using fatty acid spin labeling EPR analysis using 5-nitroxyl stearate (5-DSA, Aldrich) as a spin probe [76,77].
Figure 5. Cav-1 KO mice exhibit enhanced astrogliosis and neuronal degeneration. (A–C) Light microscopic image displaying 0.5 μm thick hippocampal sections of Cav-1 KO (A-i, A-ii), aged (B-i, B-ii), and young (C-i, C-ii) stained with toluidine blue. There is a drastic reduction in neurons within the dentate gyrus (large arrow heads) and CA1 regions (arrows) of young Cav-1 KO mice compared to young and aged WT. In addition, there...
appears to be the presence of more glia and glial scar formation within the dentate gyrus of Cav-1 KO mice as indicated by the darker gray cell bodies intermixed with the neurons. (D) Hippocampal coronal cryostat sections (10 μm) from WT and Cav-1 KO mice were stained with Nissl (neuronal marker, red pixels) and GFAP (astrocyte marker, green) to show no overlap between neurons and astrocytes. (E) Coronal cryostat sections (25 μm) of 2 month WT, 2 month Cav-1 KO and 12 month Cav-1 KO stained with 0.0004% Flouoro-JadeB and fluorescent red Nissl with DAPI. Areas from CA1 of the hippocampus were imaged. WT CA1 showed well-organized astrocytes. Two month Cav-1 KO had areas of disorganized astrocytes with lightly labeling areas of potential future plaque development. Twelve month Cav-1 KO CA1 areas had large bright, entangled green fluorescence with red neurons inside and significantly less organized astrocytes, further demonstrating a degenerating neuronal model.

doi:10.1371/journal.pone.0015697.g005

isolate hippocampal areas prior to preparation of grids. Grids (70 nm sections) were stained with uranyl acetate and lead nitrate for contrast and observed on the electron microscope [JEOL 1200 EX-II (Tokyo, Japan)] equipped with a digital camera system. 25 random low magnification micrographs of the stratum radiatum were obtained from each specimen. Micrographs were analyzed for the quantity of synapses and for synapse abnormalities (reduction or changes in synapse and dendritic filopidal spine morphology, i.e., degradation of cytoskeletal architecture). The dendritic profiles were characterized by abundant organelles such as mitochondria and endoplasmic reticulum and frequent contacts from vesicle-filled axon terminals. Spine synapses were identified by an electron dense region associated with vesicles presynaptically and that lacked cellular organelles or contained a spine apparatus (as indicated by cytoskeletal architecture) with post-synaptic densities as described previously [79,80,81,82].

Generation of SynCav1 construct

To link the neuron-specific synapsin (Syn) promoter with the Cav1 cDNA, XbaI-SalI DNA fragment containing the Syn promoter was inserted into the NheI-SalI sites of the pEGFP-N1 (Clontech) and the resulting plasmid was designated pSyn-EGFP. A 685bp Cav1 cDNA was isolated from the pCRII-TOPO vector (Invitrogen) by PmeI-NotI digest and inserted into the SmaI-NotI site of the pSyn-EGFP to generate the pSyn-Cav1, in which the EGFP gene was replaced with the Cav1 cDNA. The Syn-promoter-Cav1 cassette was isolated from the pSyn-Cav1 and inserted into the BamHI site of the HIV1 vector backbone plasmid pHIV7 [83] and the resulting plasmid was designated pHIV1-Syn-Cav1.

Statistics Analysis

All parametric data were analyzed by unpaired t-tests or ANOVA Bonferroni’s Multiple Comparison as appropriate; post hoc comparisons were made by Student Neuman Keuls tests. Significance was set at p<0.05. Statistical analysis was performed using Prism 4 (GraphPad Software, Inc., La Jolla, CA).

Figure 6. Cav-1 KO mice have reduced hippocampal synapses. Synapses were quantified by routine electron microscopy as previously described [82]. EM analysis revealed a significant reduction in hippocampal synapses in both (C) Cav-1 KO (Yg) and (B) Ag mice compared to (A) WT. Synapses are indicated by red circles in WT, blue circles in Ag, and green circles in Cav-1 KO. (D) WT micrographs exhibited dendritic processes (indicated by d) with intact cytoskeletal architecture (arrows and arrowheads), while (E) Ag and (F) Cav-1 KO displayed less organized dendritic shafts (asterisk) with more abundant astrocyte presence (arrows). (G) Quantitation of data.

doi:10.1371/journal.pone.0015697.g006
Acknowledgments

We are grateful for immunofluorescence microscopy assistance from James Feramisco (Ph.D., Professor of Medicine, University of California, San Diego, La Jolla, CA) and Kersi Pestonjamasp (Ph.D., junior faculty, University of California, San Diego, La Jolla, CA). We are grateful to Dr. Marilyn G. Farquar and the use of her electron microscopy facility at UCSD. We are also grateful for the technical support from Yue Hu (B.S., technician, University of California, San Diego, La Jolla, CA), Michael Kidd (B.S., technician, University of California, San Diego, La Jolla, CA), and Ana Moreno (B.S., technician, University of California, San Diego, La Jolla, CA).

Author Contributions

Conceived and designed the experiments: BPH PMP HHP SSA.
Performed the experiments: BPH MP TY MLP JAB IRN AM SSA.
Analyzed the data: BPH MLP IRN HHP.
Contributed reagents/materials/analysis tools: BPH DMR PMP HHP JNP JH.
Wrote the paper: BPH JMS DMR HHP.

References

1. Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA (2003) Alzheimer disease in the US population: prevalence estimates using the 2000 census. Arch Neurol 60: 1119–1122.

2. Bishop NA, Lu T, Yankner BA (2010) Neural mechanisms of ageing and cognitive decline. Nature 464: 529–535.

3. Yankner BA, Lu T, Loerch P (2008) The aging brain. Annu Rev Pathol 3: 41–66.
1. Norris CM, Halpain S, Foster TC (1998) Reversal of age-related alterations in membrane-associated IDE in brain tissue and cultured cells: Relevance to Abeta metabolism. Alzheimer Dis Assoc Disord. 12(1): 43-51.

2. Thibault O, Porter NM, Chen KC, Blalock EM, Kaminker PG, et al. (1998) Cholesterol and lipid microdomains stabilize the postsynapse at the neuromuscular junction. J Cell Biol. 143(4): 863-875.

3. Vanmierlo T, Bloks VW, van Wijk-van der Zee LC, Rutten K, Kerkhage A, et al. (2009) Alterations in Brain Cholesterol Metabolism in the APPS191177 mouse, a Model for Alzheimer’s Disease. J Alzheimers Dis. 16(3): 721–729.

4. Norris CM, Halpain S, Foster TC (1998) Reversal of age-related alterations in membrane-associated IDE in brain tissue and cultured cells: Relevance to Abeta metabolism. Alzheimer Dis Assoc Disord. 12(1): 43-51.

5. Thibault O, Porter NM, Chen KC, Blalock EM, Kaminker PG, et al. (1998) Cholesterol and lipid microdomains stabilize the postsynapse at the neuromuscular junction. J Cell Biol. 143(4): 863-875.

6. Calabrese B, Wilson MS, Halpain S (2006) Development and regulation of dendritic spine synapses. Physiology (Bethesda) 21: 38–47.

7. Guirland C, Zheng JQ (2007) Membrane lipid rafts and their role in axon guidance. Adv Exp Med Biol 621: 144–155.

8. Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, et al. (2001) CNS synaptogenesis promoted by glia-derived cholesterol. Science 294: 1354–1357.

9. Crameri A, Biondi E, Kuehnle K, Lutjohann D, Thelen KM, et al. (2006) The role of LDL-receptor-1(DHHC24) in cholesterol homeostasis, APP processing, and Abeta generation in vivo. Embry 25: 432–443.

10. Bullo D, Amin MC, Suraci EL, Zhang X, Xu H, et al. (2008) Detergent resistant membrane-associated-IAE in brain tissue and cultured cells: Relevance to Abeta metabolism. J Neurosci. 28(9): 21660–21660.

11. Guirland C, Zheng JQ (2007) Membrane lipid rafts and their role in axon guidance. Adv Exp Med Biol 621: 144–155.

12. Head BP, Patel HH, Tsutsumi YM, Hu Y, Mejia T, et al. (2008) Caveolin-1 interacts with lipid raft proteins. J Neurosci Res 84: 912–917.

13. Suzuki S, Numakawa T, Shimazu K, Koshimizu H, Hara T, et al. (2004) Caveolin-1 deficiency increases cerebral ischemic injury. Circ Res 100: 721–729.

14. Tatematsu K, Nakamura H, Matsumoto Y, Matsuda K, Nakata Y, et al. (2007) Caveolin-1 deficiency increases cerebral ischemic injury. Circ Res 100: 721–729.

15. Head BP, Patel HH, Tsutsumi YM, Hu Y, Mejia T, et al. (2008) Caveolin-1 interacts with lipid raft proteins. J Neurosci Res 84: 912–917.
64. Nishio S, Yunoki M, Chen ZF, Anzivino MJ, Lee KS (2000) Ischemic tolerance in the rat neocortex following hypothermic preconditioning. J Neurosurg 93: 845–851.

65. Gonzales RA, Brown LM, Jones TW, Trent RD, Westbrook SL, et al. (1991) N-methyl-D-aspartate mediated responses decrease with age in Fischer 344 rat brain. Neurobiol Aging 12: 219–223.

66. Tamamaru M, Yoneida Y, Ogita K, Shimizu J, Nagata Y (1991) Age-related decreases of the N-methyl-D-aspartate receptor complex in the rat cerebral cortex and hippocampus. Brain Res 542: 83–90.

67. Cai D, Qiu J, Cao Z, McAtee M, Bregman BS, et al. (2001) Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. J Neurosci 21: 4731–4739.

68. Magusson KR, Nelson SE, Young AB (2002) Age-related changes in the protein expression of subunits of the NMDA receptor. Brain Res Mol Brain Res 99: 40–45.

69. Monti B, Virgili M, Contestabile A (2004) Alterations of markers related to synaptic function in aging rat brain, in normal conditions or under conditions of long-term dietary manipulation. Neurochem Int 44: 579–584.

70. Bourasset F, Melussa O, Tremblay C, Julien C, Do TM, et al. (2009) Reduction of the cerebrovascular volume in a transgenic mouse model of Alzheimer's disease. Neuropharmacology 56: 808–813.

71. Zhu M, Gu F, Shi J, Hu J, Hu Y, et al. (2006) Increased oxidative stress and astroglial responses in conditional double-knockout mice of Alzheimer-like presenilin-1 and presenilin-2. Free Radic Biol Med 45: 1493–1499.

72. Gama Sosa MA, Gasperi RD, Rocher AB, Wang AC, Janssen WG, et al. (2010) Age-related vascular pathology in transgenic mice expressing presenilin 1-associated familial Alzheimer's disease mutations. Am J Pathol 176: 353–368.

73. Dickstein DL, Walsh J, Brautigam H, Stockton SD, Jr., Gandy S, et al. (2010) Role of vascular risk factors and vascular dysfunction in Alzheimer's disease. Mt Sinai J Med 77: 82–102.

74. Elder GA, Gama Sosa MA, De Gasperi R, Dickstein DL, Hof PR (2010) Presenilin transgenic mice as models of Alzheimer's disease. Brain Struct Funct 214: 127–143.

75. Shapira S, Sapir M, Wengler A, Grauer E, Kadar T (2002) Aging has a complex effect on a rat model of ischemic stroke. Brain Res 925: 148–158.

76. Gabbita SP, Butterfield DA, Hensley K, Shaw W, Carney JM (1997) Aging and caloric restriction affect mitochondrial respiration and lipid membrane status: an electron paramagnetic resonance investigation. Free Radic Biol Med 23: 191–201.

77. Gabbita SP, Subramaniam R, Alloch F, Carney JM, Butterfield DA (1998) Effects of mitochondrial respiratory stimulation on membrane lipids and proteins: an electron paramagnetic resonance investigation. Biochim Biophys Acta 1372: 163–173.

78. Behrens MM, Ali SS, Dao DN, Lucero J, Shookhtman G, et al. (2007) Ketamine-induced loss of phenotype of fast-spiking interneurons is mediated by NADPH-oxidase. Science 318: 1645–1647.

79. Banaji SS, Shamari K, Kimes N, Hueschen J, Birchmeier W, et al. (2003) Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. Neuron 40: 719–731.

80. Bouwman J, Maia AS, Camoletto PG, Posnania G, Rouhoo EW, et al. (2004) Quantification of synapse formation and maintenance in vivo in the absence of synaptic release. Neuroscience 126: 115–126.

81. Elia LP, Yamamoto M, Zang K, Reichardt LF (2006) p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins. Neuron 51: 43–56.

82. Head BP, Patel HH, Niesman IR, Drummond JC, Roth DM, et al. (2009) Inhibition of p75 neurotrophin receptor attenuates isoflurane-mediated neuronal apoptosis in the neonatal central nervous system. Anesthesiology 110: 813–825.

83. Yann PY, Li S, Wu J, Hu J, Zaia JA, et al. (2002) Design of HIV vectors for efficient gene delivery into human hematopoietic cells. Mol Ther 5: 479–484.