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Supplemental Information

Isoform-selective decrease of glycogen synthase kinase-3-beta (GSK-3β) reduces synaptic tau phosphorylation, transcellular spreading, and aggregation

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Supplemental Information

Table S1. Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-human tau (Y9)	Enzo Life Sciences	Cat# BML-TA3119-0025, RRID:AB_205263
Mouse anti-Tau 13	Covance	Cat# MMS-520R-500, RRID:AB_291452
Mouse anti-Tau (HT7)	Thermo Fischer Scientific	Cat# MN1000, RRID:AB_2314654
Rabbit anti-Tau (mouse and human)	Agilent	Cat# A0024, RRID:AB_10013724
Mouse anti-CP13 (p-Tau S202, T205)	Peter Davies	RRID:N/A
Mouse anti-PHF-1 (p-Tau S396, S404)	Peter Davies	RRID:N/A
Mouse anti-Alz50 (misfolded tau)	Peter Davies	RRID:N/A
Rabbit anti-PSD95 (post synaptic density)	Cell Signaling Technology	Cat# 3409, RRID:AB_1264242
Mouse anti-PSD95 (post synaptic density)	Thermo Fischer Scientific	Cat# MA1-045, RRID:AB_325399
Mouse anti-Synaptophysin (pre-synaptic vesicles)	Millipore	Cat# MAB329, RRID:AB_94786
Chicken anti-GAPDH	Millipore	AB2302, RRID:AB_10615768
Mouse anti-GAPDH	Thermo Fischer Scientific	Cat# AM4300, RRID:AB_2536381
Rabbit anti-GAPDH	Cell Signaling Technology	Cat# 2118, RRID:AB_561053
Rabbit anti-GSK-3β	Cell Signaling Technology	Cat# 9315, RRID:AB_490890
Mouse anti-GSK-3β	Cell Signaling Technology	Cat# 9832, RRID:AB_10839406
Rabbit anti-GSK-3α	Cell Signaling Technology	Cat# 9338, RRID:AB_2114897
Mouse anti-p GSK-3β/α-Tyr216/Tyr279	Millipore	Cat# 05-413, RRID:AB_309721
Rabbit anti-p GSK-3β/α-Tyr216/Tyr279	Bioss	Cat# bs-2073R, RRID:AB_10857314
Rabbit anti-pGSK-3β/α-Ser9	Cell Signaling Technology	Cat# 5558, RRID: AB_10013750
Chicken anti-GFP (green fluorescent protein)	Aves	Cat# GFP-1020, RRID:AB_10000240
Mouse anti-NeuN (neuronal marker)	Millipore	Cat# MAB377, RRID:AB_2298772
Rabbit anti-Fyn	Cell Signaling Technology	Cat# 4023, RRID:AB_10698604
Mouse anti-Cdk5	Millipore	Cat# 05-364, RRID:AB_2229170
LICOR goat anti-rabbit 680 (IRDye680RD)	LICOR Biosciences	Cat# 926-68071, RRID:AB_10956166
LICOR donkey anti-mouse 680 (IRDye680RD)	LICOR Biosciences	Cat# 926-68072, RRID:AB_10953628
LICOR goat anti-rabbit 800 (IRDye800CW)	LICOR Biosciences	Cat# 926-32211, RRID:AB_62184

LICOR goat anti-mouse 800 (IRDye800CW)	LICOR Biosciences	Cat# 926-32210, RRID:AB_621842
LICOR donkey anti-chicken 680 (IRDye680RD)	LICOR Biosciences	Cat# 926-68075, RRID:AB_10974977
Alexa Fluor 488 Goat anti-chicken	Thermo Fischer Scientific	Cat# A-11039, RRID:AB_253409
Cy3 goat anti-mouse	Abcam	Cat# ab97035, RRID:AB_10680176
Cy3 goat anti-rabbit	Abcam	Cat# ab6939, RRID:AB_955021
Bacterial and Virus Strains		
AAV CBA-eGFP-2a-hTau	Wegmann et al., 2019	RRID: N/A
Lenti CBA.tauRDP301L-YFP/CFP	Nobuhara et al., 2017	RRID: N/A
Chemicals, Peptides, and Recombinant Proteins		
Odyssey Blocking Buffer (PBS)	LICOR Biosciences	Cat# 927-40000, RRID: N/A
DAPI Fluoromount-G	SouthernBiotech	Cat# 0100-20, RRID: N/A
Experimental Models: Cell Lines		
Primary mouse cortical neurons	Laboratory of Dr. Teresa Gomez-Isla	RRID: N/A
Experimental Models: Organisms/Strains		
Mouse: GSK-3β-HK	MGH mouse breeding facility	From the laboratory of Dr. James Woodgett (Hoeflich et al., 2000). RRID: N/A
Software		
Axiovision Imaging Software	Carl Zeiss	RRID:SCR_002677
ZEN Digital Imaging for LSM 800	Carl Zeiss	RRID:SCR_013672
NIS-Elements for Nikon A1 Confocal Microscope	Nikon Instruments	RRID:SCR_014329
LICOR Image Studio Software	LICOR Biosciences	RRID:SCR_015795
CAST Stereology Software	Olympus	Cat# N/A, RRID: N/A
ImageJ	National Institutes of Health	RRID:SCR_003070
Zotero	https://www.zotero.org	RRID:SCR_013784
GraphPad Prism 6	GraphPad Prism	RRID:SCR_002798
Other		
Zeiss Axiovert 100 Inverted Microscope	Carl Zeiss	RRID: N/A
Zeiss LSM 800 Microscope	Carl Zeiss	RRID: N/A
Nikon A1 Confocal Microscope	Nikon Instruments	RRID: N/A
BX51 Microscope	Olympus	RRID: N/A
Stereotactic Frame	David Kopf Instruments	RRID: N/A

Transparent Methods

Ethics statement

The generation, care, and use of animals as well as all experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital (MGH). The animals' living conditions, including housing, feeding, and nonmedical care, were maintained by the house internal animal facility (Center for Comparative Medicine) at MGH. Mice of both genders were included in the experiments. Wild-type and GSK-3β-HK mice tissues were prepared and used for biochemical and immunostaining assays at MGH.

Animals

GSK-3β-HK mouse line described in Hoeflich et al. (2000) was kindly donated by Dr. James R. Woodgett. Mice were bred in-house and the mutation of GSK-3β was confirmed by tail DNA genotyping following previously published protocols (Hoeflich et al., 2000). Groups of 8-10-month-old gender-matched WT and GSK-3β-HK mice (N= 14 per genotype, 16 males and 12 females) underwent intracranial injections as described below. Brains from 8-10-month-old gender-matched non-injected WT and GSK-3β-HK mice (N= 5 per genotype, 5 males and 5 females) were also collected for baseline biochemical and immunostaining assays.

AAV production and intracranial injections

Production and intracranial injection of AAV were performed as described elsewhere (Wegmann et al., 2017, 2019). In brief, for efficient targeting of neurons in the CNS, the AAV vector used in this study was serotype AAV 8. The target protein expression is driven by the ubiquitous CBA promoter. The target gene encodes the fluorescent protein eGFP (enhanced green fluorescent protein as a transduction marker) and wild-type human tau separated by a translation interrupting 2a peptide (AAV8-CBA-eGFP-2a-4R-hTau). Vector was injected into the left entorhinal cortex (EC) of WT and GSK-3β-HK mice (volume: 1.5µl per animal and titer: ~$6X10^{12}$ infectious particles/ml). Vector was produced at Mass Eye & Ear (MEEI) Vector Core, Boston. Animals were anesthetized with isoflurane (~2%) and positioned on a stereotactic frame (David Kopf Instruments) with the following coordinates calculated from bregma: -4.5 mm anteroposterior, mediolateral: -4.5 mm, and dorsoventral: -1.7 mm from the brain surface). Mice were euthanized twelve weeks after AAV injection.

Primary neuron cultures

Primary neuronal cultures were derived from freshly dissected cortices from WT (N=3) and GSK-3β-HK (N=4) E14-16 embryos. Neurons were seeded at a density of $0.6X10^5$ cells per well on culture dishes pretreated coated with Poly-D-lysine. For tau propagation studies, neurons were transduced by direct addition of AAV (eGFP-2a-4R-hTau) particles to the culture medium at 7 days in vitro (7 DIV) as previously described (Wegmann et al., 2019). At 14 DIV, primary neuron cultures were carefully washed with PBS (without agitation) and fixed with 4% PFA for 15 min. Neurons were then washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, blocked with 5% normal goat serum (NGS) in PBS (RT), and then incubated with the primary antibodies overnight at 4°C in 5% NGS/PBS. For detection of tau recipient neurons (neurons that acquired hTau protein from transduced donor neurons), primary antibodies against GFP and hTau (Tau13) were used (see Key Resources Table for antibodies description). Appropriate secondary antibodies (Life Technologies, 1:1000) were applied in 2% NGS/PBS for 1h at RT in the dark. After washing the cells in PBS twice for 15 min, they were stained with DAPI (Roche, #10236276001, 1:1000), and washed again in PBS. Neurons were imaged using an Axiovert inverted microscope (Zeiss).
For tau phosphorylation analyzes, primary neurons from WT (N=5) and GSK-3β-HK (N=5) E14-16 embryos were treated as described above (tau propagation study) until 14 DIV (7 days after the addition of AAV (eGFP-2a-4R-hTau) particles to the culture medium at 7 DIV). At 14 DIV primary neurons were collected and lysed in cold PBS with protease and phosphatase inhibitor cocktail. The cells were transferred to a 2ml Eppendorf, homogenized, and incubated for 30 min on ice. Each sample was then centrifuged, and the supernatants were collected. Protein concentrations were determined using a Pierce BCA Protein Assay Kit. Whole cell lysates were electrophoresed on 4-12% Bis-Tris Novex mini gels (Invitrogen, #MAN0003679) in MES running buffer for SDS-polyacrylamide gel electrophoresis (Invitrogen). Gels were transferred onto nitrocellulose membranes (Thermo Fisher Scientific) and blocked for 1h in Blocking Buffer (Odyssey, Li-Cor, #927-40000). The membranes were probed with antibodies for anti-total tau, PHF-1, and

GSK3β (See Key Resources Table for complete information on antibodies). GAPDH was used as a loading control. Appropriate secondary antibodies (goat anti-rabbit-IRDye680, goat anti-rabbit-IRDye800, goat anti-mouse-IRDye680, goat anti-chicken-IRDye680, and goat anti-mouse-IRDye800 (Li-Cor)) were added at a 1:5000 dilution for 1h at RT and membranes were visualized using the Li-Cor Imaging system. Western blot images were then quantified using Image Studio Lite.

Tau aggregation assay in primary neurons

Lentiviral vectors encoding the mutant tau repeat domain (tauRD) constructs tauRD P301L-CFP and tauRD P301L-YFP were generated as previously described (Nobuhara et al., 2017) and added to neurons in culture. In short, primary neurons derived from E14-16 cortices of WT (N=10) and GSK-3β-HK (N=12) embryos were co-transduced with lentiviruses encoding tauRD P301L-CFP and tau RD P301-L-YFP on DIV1 and treated with rTg4510 brain extract on DIV6. Prior to application, rTg4510 brain extracts were diluted with culture medium to a final concentration of 100 µg/mL total protein and filtered through a 0.2-µm membrane filter, and 50 µL were added to each well (5 µg total protein/well). Primary neurons from WT and GSK-3β-HK mouse embryos were cultured in eight wells, of which three were randomly selected for quantification. Five 10X images were taken from each well (approximately 25% of each well) in an Axiovert inverted microscope (Zeiss) and the number of intracellular tau aggregates were counted using ImageJ (NIH). Cells were stained for DAPI and NeuN and the number of nuclei and neurons counted to verify consistent plating and transfection across both conditions.

Synaptoneurosomes preparation and analysis of tau content by Western blotting

Mice were euthanized by CO_2 asphyxiation and perfused through the heart with 20 ml of PBS. Brains were quickly removed, washed in PBS, and immediately frozen in liquid nitrogen and stored at -80°C until ready to be batch processed. Left EC from AAV-injected and from non-injected mice were processed to separate synaptoneurosomes from cytosolic fraction as previously described (Tai et al., 2012). Briefly, left EC was homogenized in Buffer A (25mM HEPES ph7.5, 120mM NaCL, 5mM KCL, 1mM $MgCl_2$, 2mM $CaCl_2$) with phosphatase inhibitor (Roche 04906845001) and protease inhibitor (Roche 11697498001). The homogenate was then passed through two 80um filters (Millipore Nylon Filter, NY8002500) and 70uL were separated for the total fraction. 70uL of distilled water and 23uL of 10% SDS were added to the separated homogenate and passed through a 27 1/2G needle 3 times to shear DNA. The remaining homogenate was passed through 5um filters (PALL Acrodisc, 4650) and centrifuged at 1000g for 10 min at 4°C. The supernatant was collected and placed in the ultra-centrifuge at 100,000g (38,000 rpm) for 45 min at 4°C. Meanwhile, the pellet was resuspended in 70uL of buffer B (50mM Tris HCL, 1.5% SDS, 1mM DTT). The pellet resuspension and the total homogenate fraction were then boiled for 5 min and centrifuged at 15min at 15,000g at 4°C. The supernatant of the pellet resuspension was collected as the synaptoneurosome fraction (SNS) and the supernatant of the homogenate fraction was collected as the total fraction. Finally, the supernatant was collected from the ultra-centrifuged samples as the cytosolic fraction (CYT). Fraction purity was confirmed by Western blot using a PSD95 specific antibody. Brain lysates from the different fractions (25 µg of protein) were electrophoresed on 4-12% Bis-Tris Novex mini gels (Invitrogen, #MAN0003679) in MES running buffer for SDS-polyacrylamide gel electrophoresis (Invitrogen). Gels were transferred onto nitrocellulose membranes (Thermo Fisher Scientific) and blocked for 1h in Blocking Buffer (Odyssey, Li-Cor, #927-40000). The membranes were probed with the following antibodies: anti-GFP, total tau, human tau, PHF-1, GSK3β, phospho-GSK-3β-Tyr216, phospho-GSK-3β-Ser9, GSK-3α, phospho-GSK-3α-Tyr279, CP13, Alz50, CDK5, Fyn, PSD95, and synaptophysin (See Key Resources Table for complete information on antibodies). GAPDH was used as a loading control. Appropriate secondary antibodies (goat anti-rabbit-IRDye680, goat anti-rabbit-IRDye800, goat anti-mouse-IRDye680, goat anti-chicken-IRDye680, and goat anti-mouse-IRDye800 (Li-Cor)) were added at a 1:5000 dilution for 1h at RT and membranes were visualized using the Li-Cor Imaging system. Western blot images were then quantified using Image Studio Lite.

Analysis of tau content by immunohistochemistry

Brain tissue to be used in immunohistochemistry was collected after mice were euthanized by CO_2 asphyxiation and perfused with 20 ml of PBS and 20 ml of 4% PFA/PBS. Brains were removed and cryoprotected in a series of glycerol solutions (10% glycerol with 2% dimethylsulfide (DMSO) in PBS for 24 h and then transferred to a solution of 20% glycerol with 2% DMSO in PBS for 24h). After cryoprotection, brains were flash-frozen in 2-methylbutane at -75°C and stored at -80°C until sectioning (Rosene et al.,

1986). Sectioning was accomplished using a freezing microtome set to a thickness of 40 µm, and horizontal sections were collected in ten interrupted series of sections so that the interval between sections within a given series was 400 µm. Series of free-floating sections were stored in 30% glycerol/PBS at -80°C until ready to be batch processed. One series of sections was selected for thionin staining and stereological analysis, which were immediately mounted on gelatin-subbed slides and allowed to air dry. The sections were then rehydrated in descending graded ethanol solutions (100% X2, 95%, 70% 50%, and distilled water) for 25 seconds per bath, and stained with thionin for 2 minutes. Slides were then dehydrated in ascending ethanol solutions (distilled water, 50%, 70%, 95%, 100% X2; 1 min per bath), cleared in three xylene baths for 5 min each, and coverslipped with Permount (Fisher Scientific). The thionin series was used to quantify neuronal numbers and regional volumes, while the remaining series were used for immunohistochemistry (IHC). Sections for IHC were stored in a solution of 30% glycerol/PBS at -20^{o}C until ready to be batch processed. For labeling of proteins of interest (such as hTau, total tau, p-Tau, GSK-3β, and others), sections were thawed and washed in PBS, permeabilized with 0.1% Triton-X, and blocked in 5% NGS/PBS. Primary antibodies were diluted in 5% NGS and were incubated overnight at 4°C (see Key Resources Table for a list of antibodies). The following day, sections were washed in PBS and incubated with secondary antibodies, washed in PBS, mounted in glass slides and coverslipped using mounting medium with DAPI (SouthernBiotech). Images were obtained using an Olympus BX51 microscope and a Nikon A1 confocal microscope. Images were processed in Nikon Elements and ImageJ (NIH).

Stereological neuronal cell counts

The number of hTau donor cells, hTau recipient cells, p-Tau (CP13- and PHF-1-positive), and misfolded tau (Alz50-positive) containing neurons in the EC was determined by counting all [GFP+], [hTau+/GFP+], [CP13+/GFP+], [PHF1+/GFP+], and [Alz50+/GFP+] neurons in the left EC using the CAST software (Olympus). The cell numbers were counted in three to four brain sections per mouse and in five to seven mice per group. For the total number of neurons in the left (injected) and right (non-injected) ECs, we outlined layers 1-6 of the medial and lateral entorhinal cortices and performed stereological counting of 20% of the neurons in the ROIs, followed by extrapolation to 100%. Counting was done on an Olympus BX51 light microscope equipped with a 20X objective.

Quantification and Statistical Analysis

Statistical analysis was performed using GraphPad Prism6. The applied statistical tests, post hoc analyses, and sample sizes (n) are defined in each figure legend. A p value < 0.05 was considered statistically significant. Sample size was chosen based on similar experiments in previously published studies (Wegmann et al., 2015, 2019). Unless otherwise noted, all data are presented as mean +\- SEM.

Supplemental References

Rosene, D.L., Roy, N.J., and Davis, B.J. (1986). A cryoprotection method that facilitates cutting frozen sections of whole monkey brains for histological and histochemical processing without freezing artifact. J. Histochem. Cytochem. *34*, 1301–1315.