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Wnt-pathway activation during the early stage of neurodegeneration in FTDP-17 mice

Martina Wiedau-Pazos^{*}, Eugene Wong, Esther Solomon, Maricela Alarcon, and Daniel H. Geschwind^{*}

Department of Neurology, David Geffen School of Medicine at UCLA, 635 Charles E. Young Drive South, Los Angeles, CA 90095, USA

Abstract

Glycogen synthase kinase-3beta (GSK-3 β), a key component of the Wnt signaling pathway, has been recognized as an important tau kinase with a potential pathogenic role in dementia. We have previously shown that GSK-3 β induced tau-hyperphosphorylation and Wnt-activation enhance tau-induced degeneration in *drosophila*. Here, we demonstrate that Wnt-activation occurs prior to three months of age in the JNPL3 mouse model of frontotemporal dementia (FTD). We observed that GSK-3 β becomes associated with insoluble tau, concomitant with the increase in the downstream Wnt-pathway component β -catenin. We demonstrate that this induces downstream Wnt signaling via the activation of nuclear transcription factors associated with β -catenin, suggesting that Wnt pathway activation is an early feature of the neurodegenerative process.

Keywords

dementia; transgenic animal model; Wnt-pathway

2. Introduction

Tau pathology is considered one of the hallmarks of a group of disorders that includes Alzheimer's disease (AD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy, Pick's disease and corticobasal degeneration, leading to the term, tauopathies, to describe these disorders [16].

Glycogen synthase kinase-3beta (GSK-3 β) is a kinase that has been implicated in insulin signaling. Its role in phosphorylation of tau linked to forms of degenerative dementia is well described [4,10,14,18,25]. GSK-3 β co-localizes with PHF in AD and enhances neurodegeneration via tau hyperphosphorylation and PHF formation in fly models of human tau expression [12,28]. When GSK-3 β is inhibited *in vivo*, a reduction of tau-phosphorylation and neurodegeneration is observed demonstrating a key role for GSK-3 β in tau-induced neurodegeneration [2,13,15,23,23].

^{*}Corresponding authors: MWP: mwiedau@mednet.ucla.edu; phone 310-206-9933; fax 310-206-8082.

6.1. Disclosure statement

(a) No conflicts of interest are present.

(b) The appropriate institutional approval for all animal experiments was obtained, UCLA Animal Research Committee approval number 2003-103.

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In addition to its role in insulin signaling and tau-phosphorylation, GSK-3 β holds a pivotal position in the canonical Wnt-pathway, where it phosphorylates β -catenin in concert with APC, Frizzled, and Axin, causing β -catenin degradation via the ubiquitin-proteasome pathway [1, 3,21,29]. When the Wnt signal is activated, and GSK-3 β is down regulated, un-phosphorylated/activated β -catenin accumulates and translocates to the nucleus where it interacts with cofactors to regulate the transcription of target genes that are involved in the cell cycle and cell survival [5]. Our previous data in *Drosophila* suggested that the role of GSK-3 β in the effects of Wnt pathway signaling via β -catenin and the role of GSK3- β in the phosphorylation of tau were independent of each other [12].

Here, we extend the observations in a fly model to a mammalian system and test the hypothesis that the association of GSK-3 β with mutant tau may interfere with the role of GSK-3 β in the canonical Wnt-pathway. We confirm the observation that GSK-3 β becomes insoluble early in the disease process, likely via its association with mutant insoluble tau. To further assess the consequence of this on Wnt signaling in vivo, we bred a P301L tau reporter mouse by crossing JNPL3 with the Fos-lacZ34Efu/J strain, which expresses lacZ in proportion to the amount of activated β -catenin. We show an increase of cytosolic and nuclear β -catenin early in the disease process, prior to the onset of significant tau-hyperphosphorylation and signs of neuronal degeneration, suggesting that Wnt pathway activation may be an initial step in the neurodegenerative process.

3. Materials and methods

3.1. Transgenic animals

3.1.1. JNPL3—Hybrid (C57BL/DBA2/SW) JNPL3 transgenic mice (Taconic), expressing the longest four-repeat isoform of the human tau protein with the P301L missense mutation, were initially obtained from Michael Hutton's laboratory [17]. Transgenic mice and non-transgenic littermates were bred by mating hemizygous JNPL3 mice with the B6 strain (Taconic). Animals were maintained on the B6 background. The *tau* transgene was genotyped by PCR between exons 1 and 5 of human tau cDNA according to published protocols [17].

3.1.2. LacZ mice—To generate a double transgenic mouse that allows the detection of activated β -catenin and Tcf/Lef expression, hemizygous JNPL3 mice were crossed with Tg (Fos-lacZ)34Efu (The Jackson Laboratory) [7] and bred onto a B6 background to create the JNPL3-lacZ strain. Offspring were genotyped after weaning, amplifying a 315 bp sequence of the transgene against a Tcrd control.

All procedures involving the animals and their care were approved by the UCLA Animal Research Committee.

3.2. Central nervous system (CNS) protein extracts

Brains and spinal cords were rapidly harvested after cardiac perfusion of the sacrificed animal with 25 ml of ice-cold phosphate-buffered saline (PBS). Whole spinal cords were collected, the brains were dissected into cortical, brainstem and cerebellar regions. The tissues were weighed, quickly frozen on dry ice and stored at -80°C . Each CNS piece was homogenized in ten volumes (v/w) of PBS containing protease and phosphatase inhibitors (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 30 mM β -glycerophosphate, 30 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate, sonicated and spun 30 minutes at 13000 RPM at 4°C . For soluble protein extract electrophoresis, the supernatants were collected; for sarcosoyl-insoluble fractions, extracts were prepared as described [9,12].

3.2. Western blotting

All samples were separated on 10% sodium dodecyl sulfate–polyacrylamide (SDS PAGE) gels, electroblotted overnight onto PVDF membrane (Bio-Rad) and probed with primary and peroxidase labeled secondary antibodies (company). Blots were developed using enhanced chemiluminescent (PierceEndogen) and band intensities were quantified using a laser scanner densitometer (Molecular Dynamics).

3.3. Antibodies

Antibodies against tau protein included monoclonal T14 and T46 (Zymed), monoclonal AT100, AT8, AT270 against phosphorylated forms of tau (Zymed)—recognizing epitopes that are phosphorylated by GSK-3 β , polyclonal GSK-3 β and β -catenin (Chemicon), and polyclonal lacZ (Cappel). For loading controls, monoclonal anti- β -tubulin antibody TUB 2.1 (Sigma) was used.

3.4. Immunohistochemistry

Brains from 6 month-old JNPL3 transgenic mice or 3, 6 and 9 month-old JNPL3-lacZ mice and an equal number of control mice were used for immunohistochemical analysis. After euthanizing the animals, they were perfused transcardially with 4% paraformaldehyde in saline and sodium phosphate buffer (pH 7.4). Frozen specimen were cut into 50 μ m floating sections on a cryotome, and stained using the Vectastain kit (Vector) with the primary antibodies as described in the Western blotting section. Sections were counterstained using cresyl violet and coverslipped.

3.5. Data analysis

Densitometry results of three Western blots were analyzed by ANOVA using SAS statistics software. Post-hoc data analysis was performed using Tukey test [26].

4. Results

4.1. Phosphorylation of tau and association of tau with GSK-3 β in the CNS of JNPL3 mice

Cerebral cortex from six-month-old female JNPL3 mice was evaluated using immunoblotting with antibody against hyperphosphorylated tau (AT100). We confirmed, as previously reported [24], that tau protein extracted from JNPL3 cortex and spinal cord exhibits a shift toward increased amounts of hyperphosphorylated tau in the sarkosyl-insoluble protein fraction when compared to CNS extracts from controls (figure 1, panel A.). In contrast, extracts from the cerebellum, which does not degenerate, do not contain AT-100 positive PHF-tau, although similar amounts of total human tau (figure 1, panel H lanes 5–8) expression are observed in cerebellum when compared to the cortex, spinal cord and brainstem. Immunoblotting of the soluble and sarkosyl-insoluble protein fractions from cortex using antibody against GSK-3 β reveals that GSK-3 β is shifted toward the sarkosyl-insoluble fraction in cortex, concomitant with tau (figure 1, panel A), suggesting a possible association between hyperphosphorylated tau and GSK-3 β in the cortex of JNPL3 mice.

To further analyze the association of tau with GSK-3 β , we performed double staining immunohistochemistry. Panels B-G of figure 1 show frontal cortex sections of the JNPL3 and non-transgenic animals labeled with antibody against PHF tau (AT100, red, panels B, E) and GSK-3 β (green, panels C, F). Varying loads of PHF tau are observed in neurons in the P301L brain, ranging from small clusters to large protein aggregates filling out the entire cell in a tangle shape (panel B). The distribution of GSK-3 β in neurons overlaps mainly with the small clusters of PHF tau (panel D, arrows), indicating that GSK-3 β is primarily found overlapping

with tau in a less soluble, pathological confirmation early in the process of forming hyperphosphorylated tau aggregates, months prior to overt neurodegeneration [17].

4.2. Total β -catenin levels are elevated in vulnerable CNS regions of the JNPL3 strain

We next tested the hypothesis that if enough GSK-3 β were compartmentalized in the insoluble protein fraction, less would be available to phosphorylate cytosolic β -catenin, potentially resulting in an increase of total β -catenin. We first evaluated CNS β -catenin levels in JNPL3 and control mice. Figure 1 (panel H) reveals that β -catenin levels are higher in CNS areas of the JNPL3 mice that are most prone to degeneration. Cortex, spinal cord, and brain stem contain higher amounts of β -catenin than the cerebellum, a region that does not degenerate in this model. Since GSK phosphorylation of β -catenin and its subsequent degradation is a key means of suppressing canonical Wnt signaling, this increase in β -catenin supported the notion that Wnt signaling may be altered.

4.3. β -Catenin elevation results in increased tcf activity *in vivo*

β -catenin exists in several cellular pools, therefore it was possible that an increase in total cytosolic β -catenin may not result in increased Wnt signaling. To directly evaluate the Wnt pathway signaling activity of β -catenin, we assessed its transcriptional activating effect in an *in vivo* system. β -catenin transduces the Wnt signal via its interaction with Tcf/Lef transcription factors, therefore we used a well-characterized Tcf/Lef reporter mouse strain, Tg(Fos-lacZ) 34Efu/J (lacZ) [6]. In this reporter strain, the β -galactosidase gene is activated in the presence of β -catenin/Tcf/Lef complex binding to its transcriptional activation site. We generated a JNPL3/tcf-lacZ reporter mouse by crossbreeding JNPL3 with the Tg(Fos-lacZ)34Efu/J strain (lacZ) [7] expressing β -galactosidase in the presence of the lymphoid enhancer binding factor 1/transcription factor 3 (LEF1/TCF3) mediated signaling pathway and activated β -catenin, thus acting as an *in vivo* readout of β -catenin/Tcf/Lef induced transcriptional activation.

Female JNPL3/tcf-lacZ mice, containing both the dominantly active tau transgene and the Tcf/Lef reporter, and Tcf-lacZ control littermates (without the tau transgene) were analyzed at ages 3, 6 and 9 months for PHF-tau and appearance of neurodegeneration, GSK-3 β and β -catenin levels and concomitant β -galactosidase expression as an indicator of Tcf activation/signaling. First, in parallel to the findings in the JNPL3 strain, increasing levels of hyperphosphorylated tau are observed between 3 and 9 months in the JNPL3-lacZ mouse cortex (figure 2, panels A, B and C) and spinal cord (figure 3, panels A, B and C) in contrast to wild type controls (figures 2 and 3, panels J, K and L). The expression of GSK-3 β remains elevated throughout the disease process (figures 2 and 3, panels D, E and F) when compared to the wild type controls (figures 2 and 3, panels M, N and O). At early disease stages, prior to a significant accumulation of PHF tau, β -catenin/Tcf/Lef levels are elevated in the JNPL3-lacZ cortex and spinal cord when compared to the wild type controls (figures 2 and 3, panels J, K and L). Wnt activation via β -catenin/Tcf/Lef diminishes with ongoing accumulation of PHF-tau during aging as demonstrated by β -galactosidase reporter expression (figures 2 and 3, panels G, H and I).

Western blotting was used to verify these histochemical observations. Figure 4.a. confirms that β -galactosidase levels are significantly increased in soluble protein extracts from the frontal cortex and spinal cord of 3 month-old JNPL3 mice compared to the cerebellum. Figure 4.b. depicts the reduced expression of β -galactosidase in the cortex as the JNPL3-lacZ mouse ages to 9 months, whereas the β -catenin/Tcf/Lef as indicated by β -galactosidase levels remained stable in the wild type control.

5. Discussion

In this study, we first confirmed previous reports that the expression of human P301L tau in the JNPL3 mouse CNS leads to the formation of PHF-tau aggregates and elevated levels of insoluble GSK-3 β in a pattern that correlates with regional degeneration in this model. High levels of PHF-tau and GSK-3 β are observed in spinal cord and cortex, whereas lower levels of GSK-3 β and PHF-tau are observed in the cerebellum. In parallel with the increase of GSK-3 β in affected CNS regions, we report the novel observation that total β -catenin levels are elevated in the same pattern in affected CNS regions early in the disease course, prior to overt pathology. In addition, we show that this elevation of β -catenin specifically reflects activated Wnt signaling, which induces Tcf transcriptional activity. This may seem paradoxical, since GSK-3 β activity is expected to diminish β -catenin levels. In turn, a reduction of β -catenin would be expected to lead to reduced Wnt signaling/Tcf transcriptional activity. However, we also find that during this early phase of disease, GSK-3 β becomes co-localized with pathologically phosphorylated tau in the insoluble protein fraction, which we hypothesized would lead to a reduced availability of GSK-3 β in the cytosol, causing elevated β -catenin due to decreased phosphorylation of β -catenin by GSK- β . We observed this elevation of β -catenin at three months in an early stage of the disease prior to overt neurodegeneration.

These findings are in accordance with an earlier invertebrate study, in which we evaluated the function of β -catenin/Tcf/Lef in both over expression and loss-of-function studies in a *Drosophila* eye model of human tau over expression. When homologs of β -catenin and the nuclear co-transcription factor Tcf were increased in *Drosophila*, the neurodegeneration phenotype was enhanced [12]. In contrast to PHF formation, which was observed only in adult flies, the exacerbation of degeneration caused by GSK-3 β occurred at all developmental stages, - larval, pupal and adult. Thus, the tangle formation took place later in the fly than the initiation of cell death. Feany and colleagues also observed cell death without NFT's in drosophila overexpressing human tau wild type and several mutants [28].

Our previous fly data suggested that increased Wnt signaling via β -catenin could be an early feature in tau related neurodegeneration. Here we tested this hypothesis in a mouse model of FTD. The present study is the first *in vivo* report providing evidence in mammals, that total and activated β -catenin, and canonical Wnt signaling via β -catenin/Tcf/Lef complex are elevated many months prior to overt pathology with neuronal loss. Since we observed that β -catenin is elevated only early in the disease process in the JNPL3 mouse, our results suggest that β -catenin may play a role in the initial phase of degenerative changes.

These findings are particularly interesting in light of previous reports in mice with conditional over expression of GSK-3 β . Aspects of tau-induced neuropathology, such as tau hyperphosphorylation, reactive astrogliosis, and neuronal death [27], and spatial learning deficit [11] were reported when GSK-3 β was over expressed. When GSK-3 β over expression was inhibited, normal GSK-3 β activity, reduced levels of phosphorylated tau, and diminished neuronal death were shown [8], supporting a more complex role of GSK-3 β in neurodegeneration than solely its role in tau-phosphorylation.

Thus, complementary to observations in the fly [12], the present evidence that β -catenin is increased early in the disease course before NFT formation peaks in the mouse supports the hypothesis that cellular processes leading to neuronal degeneration may not be initiated by tau hyperphosphorylation itself. We hypothesize that tau hyperphosphorylation is a product of increased interaction between GSK-3 β and tau, whereas the effect of this interaction on the Wnt pathway may be a separate consequence of the tau/GSK-3 β interaction, both, leading eventually to neurodegeneration. This hypothesis is supported by previous reports that the link of tau phosphorylation and GSK-3 β activity to neurodegeneration is not consistent in studies

of transgenic mice [19,20,27]. Therefore, based on the present data, we conclude that tau toxicity is not solely attributable to altered microtubule binding and its aggregation, but that tau can also indirectly affect cellular pathways causing neurodegeneration. The role of the Wnt pathway, and its connection to other processes that are implicated in neurodegeneration, such as cell cycle activation, and its interaction with other pro-degenerative factors, such as β -amyloid, as well as Wnt-pathway inhibitors such as proteins of the Dickkopf family that have been implicated in Alzheimer's disease [6], are deserving of further investigation.

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Figure 1. GSK-3β co-localizes with hyperphosphorylated mutant tau in vulnerable CNS regions of the JNPL3 mouse

Soluble (sol; lanes 1, 3, 5, 7 of blot) and sarkosyl-insoluble protein fractions (sark; lanes 2, 4, 6, 8 of blot) of P301L mice CNS extracts were blotted, and exposed to antibodies against PHF-tau (AT100) and total GSK-3β. Lane 1 and 2 were loaded with cerebellar material, lane 3 and 4 were loaded with spinal cord material, lanes 5 and 6 contain brainstem material, and lanes 7 and 8 contain frontal cortex material. The equivalent lanes 1 through 10 of the blot are also indicated under each graph. Bands corresponding to PHF-tau and GSK-3β were detected. Beta-tubulin loading control is shown on the Western blot. The graphs are aligned with the corresponding Western blot lanes and summarize the densitometry results and means of three experiments with standard errors are shown. Two y-axes were used as the numerical results for the soluble and sarkosyl-extracted materials differ. For soluble protein fractions, ratios of PHF-tau (upper graph) and GSK-3β (lower graph) to β-tubulin standard expression were calculated. Asterisks mark vulnerable brain regions that are statistically significant ($p < 0.05$ using post-ANOVA Tukey test) from the results obtained from cerebellum, which is the control region that is spared from neurodegeneration.

Abbreviations: sark, sarkosyl extract; sol, soluble extract; β-tub, β-tubulin.

Figure 2. GSK-3 β co-localizes with PHF-tau in cortical neurons

Confocal images of frontal cortical sections co-immunostained with monoclonal antibody against PHF-tau (AT180) and polyclonal antibody against GSK-3 β . Panels A and B: JNPL3 mouse; PHF-tau (red) and GSK-3 β (green), nuclear stain with DAPI (blue). Panel C: overlay of A and B, yellow arrows point to some of the co-localization areas.

Panels D and E: non-tg control mouse; PHF-tau (red) and GSK-3 β (green), nuclear stain with DAPI (blue). F: overlay of D and E. Scalebar: 20 μ m.

Figure 3. Total β-catenin expression is elevated in the vulnerable CNS regions of the JNPL3 mouse Equal amounts of soluble protein extracts from four different brain regions, cerebellum, spinal cord (S.c.), brainstem and cortex of 6-month-old JNPL3 and non-transgenic controls were loaded. The expression of total β-catenin is significantly increased in the cortex, brainstem and spinal cord of the tau mutant compared to the control (indicated by asterixes). The total Tau control (T14 antibody) indicates that this β-catenin increase in vulnerable CNS regions is independent of tau-levels. The CNS regions most vulnerable to neurodegeneration in the JNPL3 mouse, cortex, brainstem and spinal cord demonstrate elevated β-catenin levels compared to the unaffected cerebellum. Abbreviations: β-tub, β-tubulin; β-cat, β-catenin; S.c., spinal cord.

Figure 4. Increased β -catenin/Tcf/Lef in cortex and spinal cord affected by degeneration in the presence of mutant P301L tau

4.A.: JNPL3/tcf-lacZ cortex (panels A to I) and control mouse cortex (panels J to R) sections stained with antibodies against AT100 for PHF-tau (Figure 4.A. panels A to C, and J to L), GSK-3 β (Figure 4.A. panels D to F, and M to O) and β -gal (Figure 4.A. panels G to I, and P to R) demonstrate an increase in PHF tau aggregation during aging, with a relatively stable expression of GSK-3 β and significantly reduced expression of β -gal during aging. All panels show 50 μ m cortical sections of 3 month (row 1 and 4), 6 month (row 2 and 5) and 9 month (row 3 and 6) old animals. Size bar: 20 μ m.

4.B.: JNPL3/tcf-lacZ lumbar spinal cord (panels A to I) and control mouse lumbar spinal cord (panels J to R) sections stained with antibodies against AT100 for PHF-tau (Figure 4.A. panels A to C, and J to L), GSK-3 β (Figure 4.A. panels D to F, and M to O) and β -gal (Figure 4.A. panels G to I, and P to R) demonstrate an increase in PHF tau aggregation during aging, with a relatively stable expression of GSK-3 β and significantly reduced expression of β -gal during aging. All panels show 50 μ m cortical sections of 3 month (row 1 and 4), 6 month (row 2 and 5) and 9 month (row 3 and 6) old animals. Size bar: 20 μ m.

Figure 5. β -catenin/Tcf/Lef expression is elevated in the vulnerable CNS regions of the JNPL3/tcf-lacZ mouse

5.A.: Equal amounts of soluble protein extracts from four different brain regions, cerebellum, spinal cord, brainstem and cortex of 6 month JNPL3/tcf-lacZ and lacZ controls were loaded. The expression of β -catenin/Tcf/Lef is significantly increased ($p < 0.001$ post-ANOVA Tukey test) in the cortex, brainstem and spinal cord of the tau mutant compared to the control (asterixes). The total Tau levels are similar for the different regions. The CNS regions most vulnerable to neurodegeneration in the JNPL3/tcf-lacZ mouse, cortex, brainstem and spinal cord, demonstrate elevated β -catenin/Tcf/Lef levels compared to the unaffected cerebellum.

5.B.: Western blot results of cortical soluble protein extracts from JNPL3/tcf-lacZ and control mice at age 3, 6 and 9 months. The β -galactosidase levels are reduced in the JNPL3/tcf-lacZ strain as the mouse ages, but not in the lacZ control mice.

The graphs depict means and standard errors of three experiments.