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Hypothermia mediates age-dependent increase of tau phosphorylation in db/db mice

Noura B. El Khoury [a,b,1], Maud Gratuze [a,b,1], Franck Petry [a,b], Marie-Amélie Papon [b], Carl Julien [b], François Marcouiller [a,b], Françoise Morin [b], Samantha B. Nicholls [c], Frédéric Calon [b,d], Sébastien S. Hébert [a,b], André Marette [e], Emmanuel Planel [a,b,*]

[a] Université Laval, Faculté de Médecine, Département de Psychiatrie et Neurosciences, Québec, QC, Canada
[b] Centre de recherche du Centre Hospitalier de l'Université Laval de Québec, Axe Neurosciences, Québec, QC, Canada
[c] MassGeneral Institute for Neurodegenerative Disease, Department of Neurology, Alzheimer's Disease Research Laboratory, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA
[d] Université Laval, Faculté de Pharmacie, Québec, Canada
[e] Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Quebec City, QC, Canada

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ABSTRACT

Accumulating evidence from epidemiological studies suggest that type 2 diabetes is linked to an increased risk of Alzheimer's disease (AD). However, the consequences of type 2 diabetes on AD pathologies, such as tau hyperphosphorylation, are not well understood. Here, we evaluated the impact of type 2 diabetes on tau phosphorylation in db/db diabetic mice aged 4 and 26 weeks. We found increased tau phosphorylation at the CP13 epitope correlating with a deregulation of c-Jun. N-terminal kinase (JNK) and Protein Phosphatase 2A (PP2A) in 4-week-old db/db mice. 26-week-old db/db mice displayed tau hyperphosphorylation at multiple epitopes (CP13, AT8, PHF-1), but no obvious change in kinases or phosphatases, no cleavage of tau, and no deregulation of central insulin signaling pathways. In contrast to younger animals, 26-week-old db/db mice were hypothermic and restoration of normothermia rescued phosphorylation at most epitopes. Our results suggest that, at early stages of type 2 diabetes, changes in tau phosphorylation may be due to deregulation of JNK and PP2A, while at later stages hyperphosphorylation is mostly a consequence of hypothermia. These results provide a novel link between diabetes and tau pathology, and underlie the importance of recording body temperature to better understand the relationship between diabetes and AD.

1. Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the elderly, accounting for 60 to 70% of all dementia cases (*i.e.*, 47.5 million people worldwide according to the WHO 2015 report), and is thus considered one of the major public health concerns (Querfurth and LaFerla, 2010). Neuropathologically, AD is characterized by a dramatic loss of neurons in many regions of the brain, the extracellular accumulation of senile plaques composed of the amyloid-β peptide (Aβ) (Glenner and Wong, 1984), and the presence of intraneuronal neurofibrillary tangles (NFTs) consisting of abnormally hyperphosphorylated tau protein assembled in paired helical filaments (PHFs) (Grundke-Iqbal et al., 1986). Tau is a microtubule-associated protein that is abundant in the central nervous system and expressed mainly in Axons. *Tau* hyperphosphorylation has been shown to induce PHFs and tangle formation *in vitro* (Alonso et al., 2001) and is thought to be a critical event in the pathogenesis of AD since it correlates with the degree of cognitive impairment in AD (Arriagada et al., 1992; Bretteville and Planel, 2008; Duff and Planel, 2005; Tremblay et al., 2007).

Whereas only a small proportion of AD is due to genetic mutations, the large majority of cases (~99%) is late onset and sporadic in origin. The cause of sporadic AD is not fully understood due to its multifactorial components including environmental factors that interact with biological or genetic susceptibilities to accelerate the manifestation of the disease. Interestingly, data from preclinical and clinical studies suggest that pathogenic factors involved in the development of type 2 diabetes may promote the incidence of AD (Kim et al.). Indeed, several longitudinal population-based studies have detected higher AD incidence rates in diabetic patients (Arvanitakis et al., 2004; Leibson et al., 1997; Ott et al., 1999), and type 2 diabetes was shown to affect multiple cognitive functions in patients (Roriz-Filho et al., 2009). Moreover, in comparison with

* Corresponding author at: CRCHU de Québec, CHUL, P-9800, 2705 Boulevard Laurier, Québec G1V 4G2, Canada.
E-mail address: emmanuel@planel.org (E. Planel).
[1] These authors contributed equally to this work.
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age-matched control, AD patients show abnormalities in insulin and insulin receptor levels in the brain (Craft and Watson, 2004).

Over the last decade, there has been considerable interest on the impact of insulin dysfunction and diabetes on tau pathology. Studies have demonstrated that insulin is a neurotrophic factor capable of modulating tau phosphorylation both *in vitro* and *in vivo* (see El Khoury et al., 2014 for review). Moreover, we (Papon et al., 2013; Planel et al., 2007b) and others (Clodfelder-Miller et al., 2006; Jolivalt et al., 2008; Ke et al., 2009) have reported tau hyperphosphorylation in mouse models of type 1 diabetes. *Tau* hyperphosphorylation has also been reported in rat (Jung et al., 2013; Li et al., 2007) and mouse (Kim et al., 2009) models of spontaneous type 2 diabetes. In addition, Liu et al. have reported higher tau hyperphosphorylation in the brains of type 2 diabetes patients (Liu et al., 2009). However, the mechanisms underlying increased tau phosphorylation in these latter models remain unclear.

Here, we investigated tau phosphorylation and its molecular mechanisms in a spontaneous model of type 2 diabetes: the db/db mouse. This is a well-established murine model bearing a mutation in the leptin receptor, which regulates adipose tissue mass through hypothalamic effects on satiety and energy expenditure (Hummel et al., 1966). Consequently, db/db mice are hyperphagic and develop obesity associated with hyperinsulinemia and severe insulin resistance (Hummel et al., 1966). Our results showed a sustained hyperphosphorylation of tau in db/db mice as early as at 4 weeks and up to 26 weeks of age. This was associated with changes in JNK and PP2A levels in 4-week-old mice but not in 26 week-old diabetic animals. Importantly, we made the novel observation that hypothermia is a consequence of type 2 diabetes in this model and that it plays a major cause in tau hyperphosphorylation since it could be reversed following normothermia.

2. Materials and methods

2.1. Animals

Male db/db (homozygous BKS.Cg-*Dock7*^{m}+/+*Lepr*^{db}/J, stock no. 000,642) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) along with male heterozygotes from the colony used as control (db+). Mice were maintained in a temperature-controlled room (~23 °C) with a light/dark cycle of 12/12 h, and experiments were performed during the light period. All animals had access to food and water *ad libitum*. Animals were handled according to procedures approved by the Comité de Protection des Animaux du CHU under the guidelines of the Canadian Council on Animal Care.

2.2. Monitoring of physiological parameters and metabolic features

All mice were monitored for diabetes features, notably glycemia and insulinemia. Non-fasting blood glucose was measured using a glucometer with reagent strips (ACCU-CHEK ® Aviva Nano; Roche Diagnostics GmbH, Mannheim, Germany), or a Glucose Assay Kit (Biovision Inc., Mountain View, CA, USA). Plasma insulin was determined using a sandwich enzyme immunoassay according to the manufacturer instructions (Mouse Insulin ELISA, Mercodia, Sweden). The Glucose Tolerance Test (GTT) was performed by fasting the mice for 6 h (0′), and then injecting them intraperitoneally with 10% dextrose (0.1 ml/10 g). For Insulin Tolerance Test (ITT), fasted mice were injected intraperitoneally with 1 U/day of insulin (Humulin R; Elli Lilly & Co., Indianapolis, IN, USA). Blood samples were collected and glucose levels were measured at different time points (0, 15, 30, 60 and 90 min) as mentioned above. All mice were weighted at sacrifice and the body temperature was monitored using a rectal probe (Thermalert TH-5, Physitemp, Clifton, NJ, USA).

2.3. Protein extraction

Mice were killed by decapitation without anesthesia, since anesthesia can increase hypothermia-induced tau phosphorylation (Planel et al., 2007a). Brains were immediately removed and tissues were dissected on ice. Hippocampal tissues were quickly weighed, frozen on dry ice and maintained at −80 °C until they were homogenized without thawing in 5 times volume/weight of Radioimmunoprecipitation Assay (RIPA) (50 mM Tris–HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μl/ml of Proteases Inhibitors Cocktail (P8340, Sigma-Aldrich, St. Louis, MO)), using a mechanical homogenizer (TH, Omni International, Marietta, GA). Samples were then centrifuged for 20 min at 20,000 g at 4 °C. The supernatant was recovered, diluted in sample buffer (NuPAGE LDS; Invitrogen, Carlsbad, CA) containing 5% of 2-β-mercapto-ethanol, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μl/ml of Proteases Inhibitors Cocktail (P8340; Sigma-Aldrich), and heated for 10 min at 95 °C. Depending on the antibody used, 7–21 μg of protein were analyzed as described previously (Planel et al., 2001).

2.4. Production of TauC3

TauC3 was expressed as described in Barghorn et al. (Barghorn et al., 2005). A point mutation introducing a stop codon to the pET29b full-length (441 amino acids; 2N4R) Tau plasmid (addgene plasmid # 16316) at residue 422 allowed for expression of recombinant TauC3. Briefly, protein was expressed in *Escherichia coli* strain Bl21(DE3) to an O.D. of 0.6 and induced with 1 μM IPTG for 4 h at 37 °C. The pellet was re-suspended in a buffer of 20 mM MES, 1 mM EGTA, 0.2 mM $MgCl_2$, 5 mM DTT, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (sigma), pH 6.8. Cells were disrupted using sonication and boiled for 20 min at a NaCl concentration of 500 mM. The cells were then centrifuged at 127,000 *g* for 45 min at 4 °C and the supernatant was dialyzed overnight into a buffer of 10 mM HEPES pH 7, 50 mM NaCl. The dialysate was cleared by centrifugation at 127,000 *g* for 45 min at 4C and loaded onto a cation exchange column. The TauC3 was eluted using a linear gradient over 6 column volumes to a final concentration of 60% 10 mM HEPES, 1 M NaCl.

2.5. Western blot analysis

SDS-PAGE and Western blot analysis was done as previously described (Planel et al., 2001). All antibodies used in this study are listed in Table 1. Brain homogenates were separated on a SDS-10% polyacrylamide gel and then transferred onto nitrocellulose membranes (Amersham Biosciences, Pittsburgh, PA). For APP analysis, proteins were separated by NuPAGE® Novex 4–12% Bis-Tris Gel (Invitrogen, Camarillo, CA, USA). Non-specific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature and then were incubated overnight at 4 °C with antibodies directed against the specific antibody. The following day, membranes were washed 3 times and then incubated for 1 h at room temperature with the corresponding secondary antibody (Petry et al., 2014) in 5% non-fat dry milk in PBS-T, and the immunoreactive signal intensity was visualized by enhanced chemiluminescence (ECL Plus, GE Healthcare Biosciences, Piscataway, NJ). Immunoreactive bands were visualized using ImageQuant LAS 4000 imaging system (GE Healthcare Biosciences, Piscataway, NJ) and densitometric analysis was performed with Image Gauge analysis software (Fujifilm USA, Valhalla, NY).

2.6. Immunofluorescence

Tissue fixation was done according to the “cold Bouin's method” previously developed in our laboratory (Planel et al., 2004). Briefly, animals were killed by decapitation, the brain was quickly removed and immersed in ice-cold Bouin's solution (saturated picric acid, formalin, acetic acid at 15:5:1) for 24 h and embedded in paraffin blocks. Eight to 10 μm thick sections were processed for immunohistochemical analyses. Deparaffinized and hydrated sections were incubated in Target Retrieval Solution (Dako, Carpinteria, CA) at 70 °C for 25 min for enhancement of