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FTO Knockdown Decreases Phosphorylation of Tau in Neuronal Cells; A Potential Model Implicating the Association of FTO with Alzheimer's Disease

Ryan T Pitman[#], Jason T Fong, Amanda L Stone, Joseph T Devito and Neelu Puri[*#]

Department of Biomedical Sciences, University of Illinois College of Medicine, Rockford, Illinois, USA
[#]Both Authors have contributed equally to this work

Abstract

Recent genetic studies identify variants within the Fat Mass and Obesity Associated gene (*FTO*) as important contributors to the development of obesity and suggest a potential link between obesity-associated *FTO* variants and Alzheimer's disease (AD). The mechanisms of association regarding *FTO* and AD are currently unclear; however, obesity is thought to be a well known risk factor for AD. In Alzheimer's disease hyperphosphorylation of the Tau protein at certain epitopes causes the formation of neurofibrillary tangles due to microtubule collapse. AMP-activated protein kinase (AMPk) is known to phosphorylate Tau, and previous studies have proposed a relationship between *FTO* knockdown and phosphorylated AMPk (pAMPk). In this study we show that siRNA mediated knockdown of *FTO* expression in SH-SY5Y neuroblastoma cells decreases Tau phosphorylation. This novel finding suggests the potential for a cellular mechanism that may link *FTO* function with the development of AD.

Keywords: *FTO;* Tau phosphorylation; Alzheimer's disease; Obesity; AMPk

Introduction

Obesity and its metabolic consequences are well-known to have wide-ranging effects in multiple body systems. Increasing evidence supports a strong link between obesity in middle-aged and elderly individuals, and the development of Alzheimer's disease (AD) [1]. Although the pathophysiological mechanisms connecting obesity and AD are unclear, previous studies suggest a role for leptin [2], hyperglycemia [3], and mitochondrial dysfunction [4] in the pathogenesis of AD.

Variants within the *FTO* gene can contribute to the development of obesity-related traits and obesity worldwide [5], presumably through an alteration in energy intake [6-8]. It has been proposed that individuals who are heterozygous or homozygous for *FTO* variants exhibit increased expression of *FTO* transcript levels, which may be responsible for increased BMI and obesity related traits [9].

Recent studies suggest a genetic association between variants within the Fat Mass and Obesity Associated gene (*FTO*) and Alzheimer's disease (AD) [10]. *FTO* is highly expressed in the human brain [11], and a loss of function mutation may lead to structural and functional brain abnormalities in humans [12]. Specifically, one study found that *FTO* risk allele carriers have a decreased frontal lobe brain volume, when compared to non-carriers of the *FTO* risk allele [13]. Furthermore, other studies have shown decreased word fluency in obese elderly men that have a *FTO* risk allele as well [14]. Unfortunately, there is no present cellular mechanism linking *FTO* and AD. However, our study presents a potential model linking *FTO* knockdown and the development of AD.

FTO may also be functionally coupled to the BDNF-NTRK2 signaling pathway, which is known to be involved in the pathophysiology of AD and metabolic homeostasis in humans [15,16]. Furthermore, recent studies have suggested neuropeptide Y (NPY) to have a possible role in neuroprotection from AD associated factors such as amyloid beta, through its involvement with BDNF [17]. Our previous studies investigating *FTO* and energy-balance related proteins, such as NPY [18], led us to look into the effect of *FTO* on the phosphorylation of Tau. This original idea was further developed into the major focus of this model, which attempts to link *FTO* knockdown to decreased phosphorylation of Tau implicating the potential role of *FTO* in AD.

The Tau protein stabilizes and maintains the normal morphology of microtubules in neurons of the central nervous system. However, Tau proteins are not able to stabilize microtubules when phosphorylated, which can lead to the development of neurodegenerative diseases such as AD. BDNF, AMPk, and Akt are known to associate with the phosphorylation of Tau, an important component of neurofibrillary tangles and a mediator of neuronal destruction when hyperphosphorylated [16,19,20,21]. In particular, previous studies have indicated that AMPk can directly phosphorylate Tau at Ser-396 [19,22]. Other studies have demonstrated that increased levels of Akt may correspond with a significant increase in levels of total Tau and hyperphosphorylated Tau [21]. Our lab has also shown a link between *FTO* knockdown and decreased levels of both pAkt and pAMPk, which serve roles in glucose metabolism and sensing metabolic intracellular energy levels, respectively [18,22,23]. In the present study, we postulate a connection between *FTO* expression and Tau phosphorylation, through activated AMPk.

Our findings agree with previous studies, and indicate a possible connection between *FTO* variants and AD through a proposed cellular mechanism [10,13]. Overall, we hypothesize that *FTO* knockdown may play a role in the development of Alzheimer's disease, by reducing phospho-Tau levels.

***Corresponding author:** Neelu Puri, Department of Biomedical Sciences, University of Illinois College of Medicine, 1601 Parkview Avenue, Rockford, Illinois 61107, USA, Tel: 815-395-5678; Fax: 815-395-5666; E-mail: neelupur@uic.edu

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Materials and Methods

SH-SY5Y neuroblastoma cells are a well established model for the study of Tau phosphorylation and AD pathophysiology [24]. In this study, SH-SY5Y cells were differentiated as described in one of our previous publications [18]. Prior to differentiation, knockdown of *FTO* expression was performed with the use of siRNA. SH-SY5Y cells were transfected with either anti-*FTO*-siRNA from Dharmacon (Cat no: D-004159-02; Lafayette, CO) or control siRNA (Cat No: 6568S; Cell Signaling Technology, Danvers, MA) using Dharmafect 2 transfection reagent as described previously [18].

To confirm appropriate down regulation of *FTO* in both undifferentiated (naive) and differentiated cells, qPCR was performed at several time points following transfection, and one week after induction of differentiation. Immunoblotting was performed as described previously [18,25], utilizing anti-*FTO* antibody from Novus Biologicals (Cat No: NB110-59758, Littleton, CO) and anti-β-Actin (Cat No: 5441) antibody from Sigma (St. Louis, MO). Sheep anti-mouse IgG (NA931V) and donkey anti-rabbit IgG (NA934V) secondary antibodies were obtained from GE healthcare (Piscataway, NJ). While probing for Tau phosphorylation, the anti-Tau antibody (Cat No: 4019; Cell Signaling, Danvers, MA) and anti-phospho-Tau (phospho-Ser-396) antibody (Cat No: 2934-1, Epitomics, Burlingame, CA) were used. Densitometry was performed with Image J. Experiments for each condition were repeated three times, and a representative result was shown.

Results

Naive SH-SY5Y cells treated with siRNA against *FTO* exhibited 81.8% and 69.8% reduction in *FTO* mRNA expression at 48 and 72 hours, respectively (Figure 1A). Sustained knockdown of *FTO* mRNA was validated after one week of differentiation (59.3%) (Figure 1A). *FTO* mRNA expression was found to be decreased by 69% after 72 hours of transfection (Figure 1B) with a similar result seen by immunoblotting, as protein expression was found to be decreased by 41% at 72 hours (Figure 1C). To determine a possible mechanism linking *FTO* and AD, *FTO*-siRNA transfected cells were immunoblotted for the presence of Tau phosphorylation. This phosphorylation could contribute to hyperphosphorylation of Tau, which is a known precursor to the development of the neurofibrillary tangles present in AD neurons [26]. In both naive and differentiated SH-SY5Y cells, *FTO*-siRNA transfection resulted in a significant decrease in Tau phosphorylation at Ser-396 (Figure 1D) (38% and 41%, respectively).

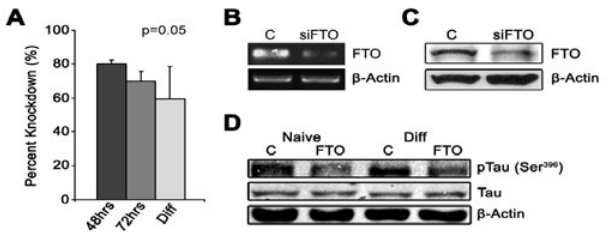

Figure 1: Down regulation of *FTO* mRNA expression in SH-SY5Y cells, and *FTO*'s effect on phospho-Tau levels. (**A**) SH-SY5Y cells were transfected with siRNA for 24 hours and then incubated for a total of 48 or 72 hours post-transfection resulting in 81.8% and 69.8% knockdown, respectively. Additionally, SH-SY5Y cells were transfected with siRNA and then differentiated for 1 week resulting in 59.3% knockdown. GAPD mRNA was used as a positive internal control. P value: p=0.05. (**B**) Down regulation of *FTO* protein expression in SH-SY5Y cells. SH-SY5Y cells transfected with siRNA for 72 hours and immunoblotted post transfection exhibited a 41% decrease in *FTO* expression. (**C**) Down regulation of *FTO* mRNA expression in SH-SY5Y cells. SH-SY5Y cells transfected with siRNA for 24 hours and then incubated for a total of 72 hours post-transfection demonstrated a 69.8% decrease in *FTO* mRNA expression. (**D**) *FTO* knockdown decreases phospho-Tau levels. SH-SY5Y cells were either transfected with siRNA and then incubated 48 hours post-transfection, or differentiated for 1 week and subsequently immunoblotted. *FTO* knockdown decreased the expression of phospho-Tau (Ser^{396}) by 38.1% in naïve and 40.8% in differentiated SH-SY5Y cells. Immunoblots are representative of at least three independent experiments.

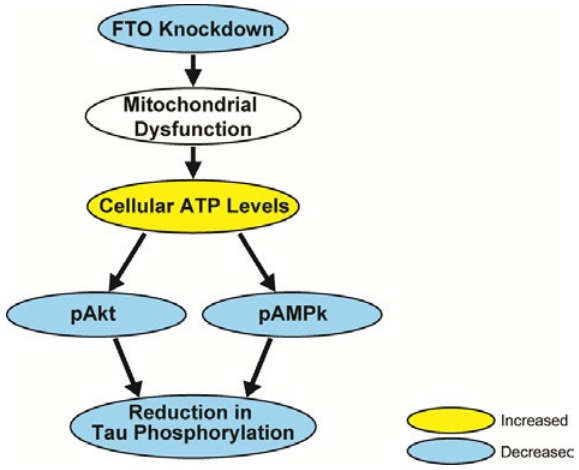

Figure 2: Proposed mechanism explaining *FTO*'s interaction with Tau: mediation of Tau phosphorylation.

FTO is highly expressed in the hypothalamus of brain, which plays a major role in the control of energy homeostasis. Tau proteins stabilize and maintain the normal morphology of the microtubules of neurons in the central nervous system. When Tau proteins are phosphorylated they no longer stabilize microtubules which results in neurodegenerative diseases like Alzheimer's disease. Decrease in *FTO* expression causes mitochondrial dysfunction and increased ATP synthesis, leading to decreased phosphorylated of AMP kinase as well as decreased phosphorylation of Akt. Knockdown of *FTO* results in reduction of Tau phosphorylation, suggesting that *FTO* may mediate Tau phosphorylation through AMP kinase and/or Akt.

Discussion

Interestingly, in the present study, *FTO* appears to regulate Tau phosphorylation. In addition, *FTO* knockdown only affects phospho-Tau (Ser-396) expression without a change in total Tau expression, suggesting a transcription-independent effect of *FTO* expression on phosphorylation of Tau. Ser-396 has been previously noted as a major epitope involved in AD [27]. Thus, when Tau is phosphorylated at this site, the protein's capabilities in microtubule binding, assembly, and stabilization are greatly diminished [28-30]. However, the mechanistic link between *FTO* gene expression and Tau phosphorylation in neuronal cells is unknown. Previous studies in our lab show a connection between *FTO* expression and the phosphorylation of AMPk and Akt in SH-SY5Y cells [18]. Other studies demonstrate that AMPk activation may contribute to Tau phosphorylation in AD patients [19]. These previous investigations indicate a potential link between *FTO* and Tau phosphorylation, through AMPk activation in AD patients.

FTO is highly expressed in the hypothalamus, an area of the brain that plays a major role in the control of energy homeostasis. Thus, we hypothesize that *FTO* has a significant role in the control of cellular energy balance, including mitochondrial function [31] and glucose homeostasis; both of which are known to be deregulated in AD [3,4]. *FTO* knockdown may be able to reduce the phosphorylation of Tau, through disruption of cellular energy balance, subsequent increases in ATP synthesis, and decreases in levels of pAMPk and pAkt. Thus, AMPk and Akt may have the potential to serve as a link in this proposed pathway of interaction between *FTO* and phospho-Tau [18,22] (Figure 2). Overall, this proposed model may give insight into one of the many

mechanisms involved in the pathophysiology of AD. However, further investigations are vital for the understanding of interactions between *FTO*, AMPk, Akt and Tau.

While *FTO* is known to be a transcriptional coactivator [32], it seems unlikely that *FTO* directly regulates the expression of either AMPk or Tau. *FTO* knockdown affects the phosphorylation of both AMPk and Akt, as shown in our previous study, without significant effects on the total levels of AMPk or Akt [18]. In the present study we found that *FTO* knockdown decreases the phosphorylation of Tau, however, there is no effect on total protein levels of Tau. It is unlikely that *FTO* co-localizes with either AMPk or Tau because *FTO* is primarily confined to the nucleus [33]. However, *FTO* codes for a 2-oxogluterate dependent nucleic acid demethylase that selectively demethylates 3-methylthymine and 3-methyluracil in single stranded DNA and RNA, respectively [34]. We speculate that *FTO* may be able to indirectly influence AMPk, Akt, Tau and other related proteins through demethylation reactions.

Additional investigations regarding the link between *FTO* variants, AD pathogenesis and other brain abnormalities, including reduced brain volume and defects in central nervous system development, will further contribute to our understanding of the involvement of *FTO* with AD. While our model is suggestive, we believe that it is a novel concept, and may impact future research concerning the relationship between *FTO*, obesity and AD. In future studies, we plan to reinforce and expand upon our proposed model, which includes investigation of other proteins that mediate down regulation of phospho-Tau through *FTO*. It is our hope that these observations will set the ground work and open new fields of research to further define the pathogenesis of AD.

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The authors have no conflicts of interest to report.

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