The regulation of pituitary-thyroid abnormalities by Peripheral administration of Levothyroxine increased BDNF and Reelin proteins expression in an animal model of Alzheimer’s disease
The regulation of pituitary-thyroid abnormalities by peripheral administration of levothyroxine increased BDNF and Reelin proteins expression in an animal model of Alzheimer’s disease

Sahreh shabani¹, Yaghoob Farbood¹, Seyyed Ali Mard¹, Alireza Sarkaki¹, Akram Ahangarpour¹*, Layasadat Khorsandi²

1. Physiology Research Center (PRC), Department of Physiology, Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran.

2. Cell & Molecular Research Center, Department of Anatomical Sciences, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Corresponding author: Akram Ahangarpour

Department of Physiology, Physiology Research Center (PRC), Ahvaz Jundishapur University of Medical Science, Ahvaz, Iran

Tel: 00989166080817

Fax: 00986133332036

E-mail address: akramahangarpour@gmail.com
Abstract
Alzheimer's disease (AD) is associated with decreased serum levels of thyroid hormones (THs), increased levels of thyroid-stimulating hormone (TSH) and decreased in protein expression of brain-derived neurotrophic factor (BDNF) and reelin in the hippocampus. In this study, we have evaluated the effect of subcutaneously (SC) administration of levothyroxine (L-T$_4$) on levels of THs and TSH as well as protein expression of BDNF and reelin in AD rats. To make an animal model of AD, Beta-amyloid (Aβ) plus ibotenic acid were infused intrahippocampally (IH), and rats were treated with L-T$_4$ and/or saline for 10 days. The levels of THs and TSH were measured by ELISA kits. Protein synthesis was detected by Western Blotting method. Results have been shown that serum level of THs, BDNF and reelin protein expression in the hippocampus were significantly decreased (p<0.001) in AD animals and elevated significantly in AD rats treated with L-T$_4$ (p<0.01). Data showed that TSH level significantly decreased in AD rats treated with L-T$_4$ (p<0.05). These findings indicated that L-T4 increased BDNF and reelin protein expression by regulation of serum THs and TSH level in Aβ-induced AD rats.

Keywords: Alzheimer's disease, Levothyroxine, Brain-derived neurotrophic factor, Reelin.

Introductions:
Alzheimer’s disease (AD) as a progressive form of neurological disorders that characterized by a cognitive decline (Boyle et al. 2003). Extracellular amyloid plaques are one of the main histopathological hallmarks of AD (Henry et al. 2010). The memory loss in AD may result from synaptic dysfunction or neuronal signaling pathway abnormalities. Indeed, Beta-amyloid peptide (Aβ) is involved in neurotoxicity and synaptic plasticity disruption during AD (Yi et al. 2016). There are findings about reelin and BDNF involvement in AD pathogenesis (Mattson et al. 2004; Pujadas et al. 2014). BDNF, a member of neurotrophins superfamily and reelin, an extracellular matrix protein, are expressed in most part of the brain, including
BDNF and reelin play an important role in neuronal survival, differentiation and synaptic plasticity in the brain (Mattson et al. 2004; Hethorn et al. 2015). Pervious results revealed a reduction of BDNF and reelin expression in the hippocampus of AD mouse models (Hock et al. 2000, Chin et al. 2007). Aβ aggregates have been reported to decrease BDNF expression (Rosa and Fahnestock, 2015). Other observations showed that reduction in reelin expression, result in precocious formation and age-related aggregation of amyloid-beta plaque (Kocherhans et al. 2010). Thyroid hormones (THs) are physiological regulators of development and differentiation of the neurons (Stenzel and Huttner, 2013). Additionally, expression reduction of amyloid precursor protein (APP) in the brain by THs may suggest a possible role for THs in AD pathology (Contreras-Jurado and Pascual, 2012). The previous reports suggested a possible link between AD and thyroid dysfunction, including increased AD risk in higher TSH levels (Davis et al. 2008). A study demonstrated that low T4 level within the normal range is a strong predictor of cognitive decline in older women (Volpato et al. 2002). Nevertheless, the exact mechanism of the connection between thyroid disease and AD risk is unclear (Van der Cammen et al. 2003). Furthermore, the reduction of reelin and BDNF protein expression has been observed in the hypothyroidism rat's hippocampus (Sui et al. 2010, Alvarez-Dolado et al. 1999). The aim of the current study was to evaluate the protective effect of peripheral L-T₄ administration on Aβ induced changes in expression of BDNF and reelin and evaluation of THs and TSH serum levels.

**Material and methods**

Beta-amyloid peptide (1-42), Ibotenic acid (Ibo) and Levothyroxine were purchased from Sigma-Aldrich Co. (Sigma-Aldrich, St. Louis, MO, USA). L-T₄ was dissolved in NaOH (0.1M) and was again diluted with different saline volume to a final concentration of 25, 50 and 100 µg/kg (Wu et al. 2011). Aβ powder was dissolved in phosphate-buffered saline (PBS,
0.01 M) and was incubated at 37°C for 5 days to form the aggregated Aβ (Zare et al. 2015). Ibo (0.5 µl at 0.6 µg/µl) was dissolved in PBS (0.01 M) and injected into DG region of the hippocampus bilaterally (Shabani et al. 2016; Ogino et al., 2014).

**Animals**

Forty eight male Wistar rats (250–300 g) were kept at room temperature (22 ± 2 °C), humidity (50±10%) and 12 h light/dark cycles with free access to sufficient food and tap water ad libitum. Experimental protocols were performed under the control of the local Ethics Committee of Jundishapur University of Medical Sciences (Ethics Code: IR.AJUMS.REC145) in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were randomly divided into six groups (8 in each):

1) Sham operated (Sh-O), 2) AD+Veh (received a bilateral DG infusion of 10ng/µl Aβ and after 48h received 0.6 µg/µl ibotenic acid at the same region of the brain, and after seven days received vehicle (saline, S.C) once a day for 10 consecutive days), 3-5) AD+L7T4 (doses of 25, 50 and 100 µg/kg, S.C once a day for 10 consecutive days, respectively), 6) ShO+L-T4 (100 µg/kg, S.C, as most effective dose, once a day for 10 consecutive days, not received Aβ and Ibo). 24h after the last injection the rats were weighed and sacrificed, the brains were removed.

**Aβ injection**

All rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride /xylazine (90/10 mg.kg⁻¹) (Sarkaki et al., 2015) and were placed in a stereotaxic frame (Narishige Co, Tokyo, Japan) and microinjected using 30-gauge needle connected to a 10 µl Hamilton microsyringe by a polyethylene tube. Microinjections of Aβ and Ibo to the dentate gyrus (DG) of the hippocampus were done with coordination of AP= −3.8 mm from the bregma, ML= ±3.5 mm, DV= −4 mm from the skull surface, according to the Paxinos and
Watson atlas (Paxinos and Watson atlas, 2006). Animals were given 7 days recovery period after surgery before SC injections.

**Assessment of Thyroid hormone levels**

After 10 days of L-T_{4} administration (SC), THs levels were determined in the blood samples of all animals. Blood samples (4 ml per animal) were collected using the cardiac puncture of animals and centrifuged at 12,000 rpm for 10 min. The obtained plasma was quickly frozen at −80 °C. The concentrations of T_{4}, T_{3}, Triiodothyronine Resin Uptake, and TSH in samples were measured by ELISA assays kits (Monobind USA Inc). The minimum detectable dose of TSH is less than 0.027 µIU/ml. This assay had high sensitivity and excellent specificity for detection of TSH. The sensitivities for T_{4} and T_{3} were 0.128 µg/dl and 0.04 ng/ml, respectively. FT_{4}I, FT_{3}I and TSH/FT_{4}I values were calculated.

**Extraction of protein**

Frozen hippocampus tissue was extracted using a radioimmunoprecipitation assay (RIPA). Buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (complete mini, Roche, Indianapolis, IN, USA). To analyze the protein fraction, extracted proteins were resuspended in 1% SDS. The concentration proteins were determined by Bradford's assay and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

**Western blotting analysis**
Hippocampus proteins were separated by SDS-PAGE on 10% acrylamide gels and were transferred onto a nitrocellulose membrane. The membranes were blocked by 5% non-fat dry milk dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST, pH 7.6) for 8hr, and then incubated overnight at 4°C with anti-reelin antibody (mouse monoclonal, dilution 1:1000; Abcam [ab78540]; USA), anti-BDNF antibody (rabbit polyclonal, dilution 1:1000; ab205067, Abcam, USA) or anti-beta Actin antibody (mouse monoclonal, dilution 1:5000; Abcam, USA). Then, after five washes with TBST, membranes were incubated with a rabbit polyclonal secondary antibody to mouse IgG HRP (dilution 1:5000) for 1hr at room temperature. The labeled proteins were detected using a chemiluminescence Western blotting system. The expression of proteins was semi-quantified by Image J analysis software and the values were normalized to β-actin.

**Statistical analysis**

Data are presented as the Mean ± SEM. All data were analyzed with one-way ANOVA and followed by Tukey’s post hoc test. The statistical significance was reflected at P values of less than 0.05.

**Results:**

**Effect of Aβ and treatment with L-T4 on body weight of rats**

As shown in Fig.1, the body weight in AD animals was significantly decreased compared with the Sh-O and Sh-O+L-T4 groups (P<0.01), and increased after S.C. injection of different L-T4 doses in AD-treated animals compared with the AD group (P<0.01).

**SC injection of different doses of L-T4 associated with higher T4 and T3 and lower TSH in the serum of AD rats.**
As illustrated in Table 1, T\textsubscript{4} and T\textsubscript{3} levels were decreased in AD rats compared with the Sh-O and Sh-O+L-T\textsubscript{4} groups (P<0.01 and P<0.001, respectively). Also, these hormones significantly increased in SC administration of 50 and 100 µg L-T\textsubscript{4} in AD rats compared with AD rats (P<0.01 and P<0.001, respectively). In AD rats, compared to the ShO and ShO+L-T\textsubscript{4} groups, serum level of TSH was significantly decreased (P<0.01, Table 1).

As illustrated in Table 1, TSH/FT\textsubscript{4} value were significantly increased in AD rats compared with the Sh-O and Sh-O+L-T\textsubscript{4} groups (P<0.001). It also significantly declined in AD-L-T\textsubscript{4} treated rats compared with AD (P<0.001).

**Different doses of L-T\textsubscript{4} (S.C.) increased hippocampus protein expressions of reelin and BDNF in AD rats.**

The expression of reelin and BDNF proteins in AD animals were decreased significantly compared with the ShO and ShO+L-T\textsubscript{4} groups (P<0.001 and P<0.001, respectively). The expression of reelin and BDNF were significantly increased in S.C. administration of 50 and 100µg L-T\textsubscript{4} in AD rats compared with AD rats, (P<0.01 and P<0.001, respectively) (Fig. 2A-B).

**Histological evaluation**

In the present study by using the Hematoxylin and Eosin (H&E) staining, the brain tissue was evaluated for Aβ plaques. These plaques were gradually diffused in the overall brain after AD induction as shown in Fig. 3 (C, D, E and F). Treatment with L-T4 improved the histological damage in a dose-dependent manner and decreased the Aβ plaques in periventricular area of brain (D, E and F in Fig. 3B) compared to C section from AD group.

**Discussion**

The results of the present study indicated that injection of Aβ1-42 into the DG caused to loss of body weight, decrease of serum levels of THs as well as a reduction in BDNF and reelin.
protein expressions in AD rats. The S.C administrations of L-T4 associated with higher T4 and T3 levels in AD rats and gained their body weight. In addition, the L-T4 administration increased BDNF and reelin expression in AD rats.

Our finding suggested that bilateral Aβ injection in the hippocampus caused reduction in body weight and the result is in agreement with previous study (Intebi et al. 2002).

The progressive deposition of Aβ in the form of senile plaques increased oxidative damage and neurotoxicity. Recently, evidence indicated that Aβ induced-toxicity is mediated by the intracellular accumulation of ROS and apoptosis (Bastianetto et al. 2006; Liu et al. 2011). Thus, Aβ accumulation is documented as one of the main bases of AD pathology (Zare et al. 2015). In our recently study have been shown that administration of Aβ1-42 into the DG resulted in spontaneous discharges impairment of neurons and the memory process disorder (Shabani et al, 2016).

Experimental studies showed that the CNS has severe requirements for THs in the brain. The concentrations THs tend to be kept within a narrow range even in the presence of extreme fluctuations of circulating T4 level (Dratman et al. 1983). Thus, even small changes in the brain level of THs may have important behavioral effects (Loosen et al. 1992). Some studies have demonstrated a relationship between thyroid function with the pathogenesis of AD, including Aβ deposition and neuronal apoptosis. As, Thyroid response elements (TREs) were found in the APP gene and T3 repressed APP in cultured neurons of rat (Contreras-Jurado et al. 2012). Volpato and colleagues showed a decline in the level of thyroxine in older women (Volpato et al. 2002). Our results showed that Aβ injection in the hippocampus caused a significant decrease in serum T4 and T3. Besides, there is a possible relationship between TSH levels and the risk of AD (Luboshitzky et al. 1996). Our findings revealed that TSH was not significant differences between the AD groups and sham groups and the result is in agreement with previous studies (Lampe et al. 1988). The serum half-life of TSH is
approximately 1 hour whereas half-life of free thyroxine is approximately 1 week, it was revealed that TSH remains within normal range in patients with pituitary hypothyroidism (Raj 2014) similar to the present results, but TSH/FT₄I value had increased three times in AD rats and declined in SC administration of L-T₄. Davis showed a higher probability of dementia among individuals with elevated TSH levels (Davis et al. 2008). In the elderly cohort study, has been shown that high TSH associated with an increased risk of AD (Forti et al. 2012). However, a decrease in TSH secretion suggests that the site of action is on the pituitary and/or hypothalamus glands (Ahangarpour et al. 2016). It was shown that high and high-normal thyroid function is associated with increased dementia risk (Chaker et al. 2016).

There are several controversial studies such as Chen and coworkers showed that serum TSH levels in AD patients were lower than those in normal control (Chen et al. 2013). In addition, several lines of evidence in patients with AD showed significantly lower T3 levels and a blunted TSH response to thyrotropin releasing hormone (TRH) (van Osch et al. 2004; Thomas et al. 1987).

Hippocampus is a recognized region for its high degree of synaptic plasticity. This region has the highest neuroanatomical expression of BDNF in the brain (Lee et al. 2009; Numakawa et al. 2010). Various study showed that BDNF regulated the phosphorylation trafficking and expression of N-methyl-D-aspartate receptor (NMDAR) subunits. BDNF facilitated Ca²⁺ influx and activated intracellular signaling pathway involved in LTP using direct implications on NMDAR (Numakawa et al. 2010; Wang et al. 2014; Mulholland et al. 2008). It has been suggested that Aβ-associated neurotoxicity may be a consequence of BDNF deficiency (Meng et al. 2013). Buchman et al. showed that levels of BDNF expression were lower in individuals with pathologic AD (Buchman et al. 2016). Furthermore, higher brain BDNF expression associated with slower cognitive decline and might also reduce the deleterious effects of AD
pathology on a cognitive decline. It was reported that cognitive deficits exhibited by mouse models of AD can be rescued by a BDNF delivery (Nagahara et al. 2009).

Reelin regulates synaptic function and plasticity in the mature brain, thereby favoring memory formation (Botella-López et al. 2009). Over expression of reelin in neurons increases the number of synapses in the hippocampus (Pujadas et al. 2014). In contrast, mice that showed declined reelin level, have fewer hippocampal synapses (Niu et al. 2008). Functionally, reelin increased LTP and protected against Aβ- induced defects (Durakoglugil et al. 2009).

A pervious finding revealed a reduction in reelin expression in the entorhinal cortex of mouse models of AD (Chin et al. 2007). Stranahan et al. 2011 showed that the protein and mRNA level of reelin decreased in an entorhinal cortex of aged rats that are cognitively impaired. In Consistent with these findings, we showed that reelin and BDNF proteins expression decreased after induction of AD by Aβ. Another study showed that reduction in expression of reelin in AD mice accelerated amyloid-Plaque formation (Chin et al. 2007) and reelin delayed amyloid-beta fibril formation and rescued cognitive deficits in a model of AD (Pujadas et al. 2014).

BDNF plays a key role in modulating synaptic transmission and plasticity (Mattson et al. 2004; Murer et al. 2001). The THs undertake important actions in the processes of neuronal maturation and migration (Luboshitzky et al. 1996). Blanco et al. 2013 reported that the decrease of T₄, T₃ and FT₄ serum levels down-regulated mRNA expression of BDNF in the hippocampus in rat. Moreover, Transient postnatal treatment of rats with THs increased hippocampal BDNF mRNA levels (Luesse et al. 1998). It was shown that T₃ administration increased reelin and BDNF proteins expression in the hippocampus (Sui et al. 2010). The significant down-regulation of reelin seen under THs deficiency was restored on T4 treatment, suggesting a relationship between thyroidal status and molecular cues that governs
neuronal migration (Pathak et al. 2011). The present study indicated; for the first time, that L-T4 administration increased expression of BDNF and reelin in AD rats.

**Conclusions**

The results of this study demonstrated that Aβ caused pituitary-thyroid abnormalities in Aβ induced AD rats, including declines of THs and a tendency to increase the serum TSH levels. These abnormalities associated with a decrease of BDNF and reelin protein expression in AD rats. Treatment of AD rats with L-T4 increased expression of BDNF and reelin using the regulation of serum THs and TSH level. Further studies are needed to clarify the neuroprotective mechanism of L-T4 on hippocampal of AD rats.

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Figure captions

Fig. 1. Effect of Aβ and treatment with L-T4 on body weight of rats (**P<0.01 vs. ShO and ShO+ L-T4, ##P<0.01 vs. AD + vehicle, n=8, one way ANOVA followed by Tukey’s Post hoc test).

Fig. 2. Effect of Aβ and treatment with L-T4 on BDNF (A) and reelin (B) protein expression in different groups. The results showed that 50 and 100 µg doses of L-T4 increased BDNF and reelin expression in AD rats. (***P<0.001 vs. Sh-O and Sh-O+ L-T4, ###P<0.01 and ###P<0.001 vs. AD + vehicle, $\text{P}<0.05$ and $$\text{P}<0.01$ vs AD+ L-T4 (25), n=8, one way ANOVA followed by Tukey’s Post hoc test).

Fig. 3. 3A: Effect of Aβ and treatment with L-T4 on the number of plaques in periventricular area 3B: Effect of Aβ and treatment with L-T4 on histopathology of the periventricular area (shown by the arrow, H&E, scale bar = 100 µm). (A) ShO, (B) ShO+L-T4(100) , (C) AD+vehicle, (D) AD+L-T4(25), (E) AD+L-T4(50), (F) AD+L-T4(100). ***P<0.001 vs. Sh-O and Sh-O+ L-T4, ##P<0.01 and ###P<0.001 vs. AD + vehicle, $$\text{P}<0.01$ vs AD+ L-T4 (25), n=5, one way ANOVA followed by Tukey’s Post hoc test.
## Table 1. Serum level of thyroid hormones and TSH in different groups

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<th>Factors Groups</th>
<th>T₄ (µg/dl)</th>
<th>FT₄I</th>
<th>T₃ (ng/dl)</th>
<th>FT₃I</th>
<th>TSH (µIU/mL)</th>
<th>T₃ uptake</th>
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<td>Sh-O</td>
<td>12.52±0.6</td>
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<td>28.45± 2.80</td>
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<td>40.72±0.04</td>
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<td>Sh-O + LT₄</td>
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<td>5.53 ±0.10</td>
<td>35.61 ±1.81</td>
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<td>22.92 ±1.66</td>
<td>40.78±0.06</td>
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<td>AD+vehicle</td>
<td>5.40± 0.055 ***</td>
<td>2.17±0.22***</td>
<td>10.7 ± 1.9***</td>
<td>2.18 ±0.22***</td>
<td>26.25 ±1.69</td>
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<td>AD+L-T₄(25)</td>
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<td>17.26 ±1.29###***</td>
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AD= Alzheimer's disease; L-T₄= Levothyroxine; thyroxine (T₄), free thyroxine index (FT₄I), triiodothyronine (T₃), free triiodothyronine index (FT₃I), thyroid stimulated hormone (TSH)

(*P<0.05, **P<0.01 and ***P<0.001 vs. Sh-O and Sh-O +L-T₄, #P<0.05, ##P<0.01 and ###P<0.001 vs. AD+vehicle, $P<0.01 and $$P<0.001 vs. AD+L-T₄(25), n=10, one way ANOVA followed by Tukey’s Post hoc test).
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