Estrogens still represent an attractive therapeutic approach for Alzheimer's disease

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Alzheimer's disease (AD) is a progressive neurodegenerative condition that goes from mild cognitive impairment in prodromal disease to severely disabling deficits in advanced stages. The risk for AD development, as well as progression and severity, clearly differ between men and women (Pike, 2017). Epidemiological studies have shown that there is a significantly increased prevalence in the development of AD in women compared to men, which is usually explained by the longer lifespan of women. This increased frequency may be due to the interplay between age and sex, in which genetic factors together with hormonal and metabolic patterns play a crucial role. Moreover, cognitive impairment has been confirmed to be greater in women than in men at the same stage of AD, likely due to reduced estrogen levels in post-menopausal women (Laws et al., 2016).

From the beginning of the 1990s, when the first evidence of a beneficial role of sex hormones on cognition appeared, to today, the interest of estrogens as neuroprotective agents for AD has had highs and lows due to a large disparity of data in the literature. Thus, excellent results have been obtained from basic and epidemiological studies but these have not been confirmed by clinical trials. In fact, the first clinical trials, in contrast to what was expected, have seen an increased risk of dementia in post-menopausal women who have undergone hormone replacement therapy (Shumaker et al., 2003).

In AD, estrogen neuroprotection seems to be exerted at multiple levels. Despite their classical protective action against neuroinflammation, synaptotoxicity and oxidative stress, recent findings demonstrate that estrogens are able to modulate the production of the two protagonists of the disease: amyloid-β (Aβ) and Tau protein.

Aβ derives from the amyloid precursor protein (APP) through β site APP cleaving enzyme 1 and γ-secretase processing that generates multiple C-termini, most ending at residue 40 and 42. $A\beta_{42}$ aggregates more quickly and stably than $A\beta_{40}$ through sequential phases: first Aβ monomers aggregate into soluble oligomers that then form insoluble oligomers, generating protofibrils and fibrils. It is well known that Aβ is a target of estrogen action at the synthetic and degradative level (Merlo et al., 2017). Moreover, numerous studies have shown that estrogens are able to shift cellular metabolism towards the non-amyloidogenic pathway, and in this case the first cut on APP is operated by the alpha-secretase with the production of a small fragment (p3) which has no ability to aggregate.

More recently, the involvement of estrogens in neuroprotective mechanisms that target Tau protein has been extensively studied. Tau is a microtubule-associated protein characterized by multiple highly regulated phosphorylation sites. The dysregulation of Tau phosphorylation leads to accumulation of its hyperphorylated form, which aggregates and forms intracellular deposits, named neurofibrillary tangles. It has been demonstrated that 17β estradiol promotes Tau dephosphorylation *in vitro* in rat cortical neurons and neuronal cells in an estrogen receptor-mediated and dose-dependent manner. Also *in vivo* studies have shown that estrogenic treatment activates signal pathways that lead to an inhibition of kinases such as GSK3beta and therefore to a reduction in Tau-phosphorylation (Munoz-Mayorga et al., 2018).

Since Aβ and Tau could be targeted by estrogens at different levels, the most recent literature in the field has dedicated attention to the relationship to Aβ/Tau interactions, and thus, *in vivo*, the two proteins are reciprocally involved in pathological signals. Several data support the amyloid hypothesis: accumulation of Aβ peptides is the primary and early event that induces neuronal degeneration, characterized by altered and aggregated Tau.

We have developed a powerful system based on mice expressing the wild-type human Tau (hTau) which were subjected to intraventricular injections of Aβ peptides, in nanomolar concentration. We discovered that $A\beta_{42}$ monomers, but not oligomers are able to produce PHF-like conformation of Tau protein, and to induce two phosphorylated epitopes which are not present in normal Tau (Ser396 and Ser422) through the activation of GSK3β, JNK and ERK 1/2 kinases in male hTau mice (Manassero et al., 2016).

Thus, we demonstrated that the intracerebroventricular injection of 200 nM $A\beta_{42}$ is not able to determine this effect in young female mice but also after ovariectomy. The same result was obtained by evaluating the total Tau protein levels.

We also showed that the treatment with $A\beta_{42}$ induces phosphorylation of the pathological sites in male and ovariectomized female mice, while controlled female's phosphorylation of the sites is not observed. To confirm whether the presence of estrogens is involved in the different effect exerted by the treatment with $A\beta_{42}$ on the pathological conformational change of Tau, groups of female mice, ovariectomized or not, were subcutaneously treated with estradiol (1 μg/kg) and fed with a phytoestrogens free diet for 3 weeks. As expected, oophorectomy significantly decreases circulating estradiol levels, whereas the treatment with estradiol completely protects both the pathological conformational change and the increase of total Tau mediated by $A\beta_{42}$ in ovariectomized females.

The enrichment with estradiol is also followed by complete protection of $A\beta_{42}$-mediated phosphorylation, after oophorectomy, of pathology-related sites. Finally, to further confirm the role of estradiol on the pathological conformation change and hyperphosphorylation of Tau, we also treated male mice with estradiol and found that this treatment is able to completely protect both the conformational change and the hyperphosphorylation of Tau (Guglielmotto et al., 2020).

Literature data indicate that estradiol treatment, at least during the early stage of AD pathology, significantly promotes the recovery of cognitive function and upregulated neurogenesis-related mediators in $A\beta_{42}$ mice and that these effects may have been due, at least in part, to decreased levels of oxidative stress (Nilsen, 2008). Thus, we tested the total antioxidant capacity and found that ovariectomy is capable of causing a significant decrease in antioxidant capacity and the simultaneous intracerebroventricular injection of $A\beta_{42}$ induces a further deterioration of the parameter. Treatment with estradiol protects the drop in antioxidant capacity by bringing it back to control values, confirming an antioxidant role of estradiol in our experimental model (Guglielmotto et al., 2020).

Finally, we measured levels of miR-218, since recent discoveries demonstrate that estrogen receptors are able to modulate the expression of microRNA involved in Tau phosphorylation (Xiong et al., 2015). In particular, it has been found that an increase of miR-218 reduces the level of target protein tyrosine phosphatase α with consequent enhancement of Tau phosphorylation.

We observed that levels of miR-218 are significantly higher in ovariectomized female mice, injected or not with $A\beta_{42}$, whereas the estradiol treatment is followed by a total protection of the miRNA increase.

The fact that the regulation of miRNAs plays a role in many pathological conditions of the central nervous system may open new windows for the research on the role of estrogens in AD.

Biological complicity of miRNA is only shortly known but it is now quite evident that these short RNAs have an important role in modulating and regulating gene expression. In the literature, there is a lot of data regarding the role of estrogens in the regulation of miRNA in cancer studies while the role of estrogen regulation in the brain is still largely unexplored. The first study of this field by Rao and collaborators (2013) showed that estradiol is able to regulate target miRNA in age and tissue specific way in ovariectomized rats. Furthermore, prolonged estrogen deprivation leads to a loss of

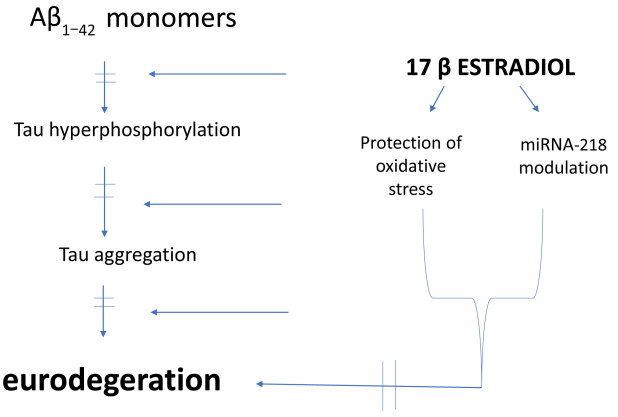

Figure 1 | **Inhibition of amyloid-β (Aβ) mediated pathological conformation of Tau by estradiol treatment.**
Estradiol replacement protects against the pathological conformation of Tau mediated by monomers of $Aβ_{1–42}$. The hypothesized mechanism is mediated both by its antioxidant activity as well as by its ability to modulate the expression of miRNA-218 linked to Tau phosphorylation.

estrogen control over miRNA regulation and less response to estrogen reintroduction. All these observations suggest that the better understanding of the mechanisms through which estrogens regulate miRNA may represent a new therapeutic strategy and could help to explain the opposite results obtained with hormone replace therapy (HRT) (Zhou et al., 2020).

Our findings suggest that hormone replacement therapy could have beneficial effects against cognitive decline. However, some studies did not report encouraging results, even finding an increased risk of dementia in post-menopausal women subjected to HRT. At this point, it becomes essential to understand the reasons for these conflicting results and "the critical window hypothesis" has been formulated. Therefore, more recent studies have been carried out to verify alternative methods of hormonal therapies, mainly in terms of time of intervention. Thus, studies have confirmed a clear relationship between estrogen intervention and distance from menopause, indicating a significant decrease in AD risk in women starting the HRT soon after menopause and, in any case, not after 5 years after menopause. Therefore, these results show that, in older women in whom hormone therapy was started long after the onset of menopause, an increased risk of developing the disease was observed. Conversely, cognitive impairment and even formation of neuritic plaques were instead inhibited by hormone therapy initiated in times very close to menopause (Zhou et al., 2020). However, it is also fair to state that not all authors embrace the protective role of HRT as reported in Cochrane's reviews. HT was shown to work for AD but only for 2–3 months in more recently menopausal women (Hogervost et al., 2009) but when HT is given for too long, when neurons start showing mitochondrial and calcium channel damage estrogen will accelerate that process. Obviously, these side effects must be taken into serious consideration and further studies should focus on alleviating such side effects. Based on their results, HRT cannot be recommended for cognitive improvement or maintenance in healthy postmenopausal women (Hogervost et al., 2009).

In conclusion, we can affirm that basic research on estrogen neuroprotection is crucial to reinforce the knowledge toward the potential use of estrogens as therapeutic approach for AD. Scientific explanations for the negative results on the use of estrogens obtained by clinical trials certainly suggest that estrogens may still be considered an important therapeutic target. The discouraging results may serve to better design clinical protocols in order to identify the best way for the estrogenic use in therapy to arrive at designing more and more personalized therapies. In this connection, individual factors such as age, reproductive stage, hormone levels and the interplay with other risk factors should be considered in women, in order to identify the best appropriate treatment with estrogens in the prevention of cognitive impairment.

AD begins many years before symptoms and therefore we must take advantage of this long window to modify and modulate all known risk factors for the disease. In this context estrogens and the hormonal patterns can be an important therapeutic target to develop personalized therapies, attacking the disease on several fronts.

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