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EFFECTS OF TRANSTHYRETIN ON THYROXINE AND β-AMYLOID REMOVAL FROM CEREBROSPINAL FLUID IN MICE

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ABSTRACT

Transthyretin (TTR) is a binding protein for the thyroid hormone thyroxine (T_4), retinol and β-amyloid peptide. TTR aids the transfer of T_4 from the blood to the cerebrospinal fluid (CSF), but also prevents T_4 loss from the blood-CSF barrier. It is however, unclear whether TTR affects the clearance of β-amyloid from the CSF. This study aimed to investigate roles of TTR in β-amyloid and T_4 efflux from the CSF.

Eight weeks old 129sv male mice were anaesthetized and their lateral ventricles were cannulated. Mice were infused with artificial CSF containing ^{125}I-T_4 / ^{3}H-mannitol, or ^{125}I-Aβ40 / ^{3}H-inulin, in present or absent of TTR. Mice were decapitated at 2, 4, 8, 16, 24 minutes after injection. The whole brain was then removed and divided into different regions. The radioactivities in the brain were determined by liquid scintillation counting.

At baseline, the net uptake of ^{125}I-T_4 into the brain was significantly higher than that of ^{125}I-Aβ40, and the half time for efflux was shorter (^{125}I-T_4: 5.16, ^{3}H-mannitol: 7.44; ^{125}I-Aβ40: 8.34, ^{3}H-inulin: 10.78; mins). The presence of TTR increased the half time for efflux of ^{125}I-T_4 efflux, and caused a noticeable increase in the uptake of ^{125}I-T_4 and ^{125}I-Aβ40 in the choroid plexus, whilst uptakes of ^{3}H-mannitol and ^{3}H-inulin remained similar to control experiments.

This study indicates that thyroxine and amyloid peptide effuse from the CSF using different transporters. TTR binds to thyroxine and amyloid peptide to prevent the loss of thyroxine from the brain and redistribute amyloid peptide to the choroid plexus.

Key words: choroid plexus, TTR, thyroxine, β-amyloid, efflux, CSF, Alzheimer's Disease

1. INTRODUCTION

Transthyretin (TTR), a highly conserved tetrameric protein of 55 kDa, is synthesized mainly in the liver in the system and is also present in the choroid plexus (CP) and retina and in the cytoplasm of ependymal cells of brain ventricles [1]. The CP contains 10 times more TTR mRNA in per gram of tissue than liver and secretes TTR at rate 13 times greater than liver [2]. TTR production accounts for

at least 20% of all protein synthesised by the CPs in the rat, and contributes approximately 50% of all secreted proteins into the cerebrospinal fluid (CSF) [3,4]. About 6% of total protein in CSF is TTR (~15 μg TTR mL^{-1} of CSF), which binds 80% of T_4 in the CSF [5].

The secretion of TTR by the CP into CSF has been suggested to play a key role in the transfer of T_4 from the blood to the CSF, as a unidirectional secretion of TTR to the CSF may drive thyroxin hormones (TH) across the blood-CSF barrier (BCB) [6]. In humans, TTR transports about 15% of plasma T_4, and the rest of T_4 are carried with other TH distributing proteins, e.g. thyroxine binding proteins (TBG), albumin [7]. In TTR knockout (KO) mice, TTR absence does not impair TH tissue contents, despite the low circulating levels of total T_4 [8,9]. These observations are in agreement with the free hormone hypothesis, that the hormone biological activity depends only on its free plasma concentration [10]. In the CSF, TTR is a major T_4 carrier [5]. It was reported that T_4 distribution from the CSF into the brain is TTR-dependent and occurs via receptor-mediated endocytosis [11,12]. The TTR-T_4 interaction poses a preferential relevance in the brain than in the peripheral circulation, given there are two other T_4 transporter proteins besides TTR being present in plasma with TBG having the highest affinity for T_4, whereas TTR is the major carrier for T_4 in the CSF.

Besides being the carrier of T_4 and retinol, TTR has been suggested to bind β-amyloid peptide (Aβ) essentially protecting the brain from neurotoxic accumulations called amyloid plaques, a hallmark of Alzheimer's disease (AD) [13-16]. TTR binds to both Aβ monomers and oligomers, but preferentially binds to the latter [17]. TTR suppressed Aβ aggregation *in vitro* via an interaction between the thyroxine binding pocket of the TTR and Aβ residues 18-21 [14], and prevents its toxicity [18]. In rat CP epithelial cell culture experiments, TTR was found to sequester Aβ in the CSF and guided Aβ to the CP for enzymatic degradation and removal [19, 20]. TTR is suggested as a protease with apolipoprotein A-I (apoA-I) and Aβ as substrates [21]. TTR cleaves Aβ in multiple positions with some of the generated Aβ peptides displaying lower amyloidogenic potential than the full-length counterpart [18]. As TTR was able to degrade aggregated forms of Aβ, it may contribute not only to the maintenance of Aβ levels within a normal range, but also to the removal of deposited Aβ in case of imbalance and disease.

TTR-Aβ interaction seems to have a neuroprotective effect in AD patients. TTR levels in the brains of AD patients are negatively correlated with the abundance of amyloid plaques [22], and TTR is upregulated in response to elevated Aβ levels in transgenic mice overexpessing mutant amyloid precursor protein [23]. Significant reduction of TTR concentration in CSF of AD patients, compared to normal age controls was found by a number of studies [24-27], suggesting the AD patients either having reduced TTR production or depleting TTR with enhanced level of Aβ in the brain. While Schultz et al. [28] did not find significant changes in TTR in CSF between AD patients and normal controls, but found a significant reduction in CSF TTR (14%) in AD patients who were medicated with cholinesterase inhibitors compared to those AD patients who were not.

This study was to characterize roles of TTR on β-amyloid clearance from CSF in comparison to thyroxine (T_4) using an *in situ* mouse brain perfusion technique, and unravel mechanism of both T_4 and Aβ efflux in a physiology model.

2. RESULTS

2.1. Baseline distribution of ^{125}I-T_4 / ^{125}I-Aβ40 in the brain

At baseline, levels of both ^{125}I-T_4 and ^{125}I-Aβ40 in the brain were significantly higher than their size matched extracellular markers (mannitol and inulin), suggesting active uptakes of T_4 and Aβ40 (Fig 1A, Fig 2A). Regionally, net levels of ^{125}I-T_4 and ^{125}I-Aβ40 in the CP, EP, were higher than those in FX and CB, presumably the latter tissues were further away from the injection site – the lateral ventricles (Fig 3,4).

2.2. Efflux half time of ^{125}I-T_4 / ^{125}I-Aβ40

Levels of ^{125}I-T_4, ^{125}I-Aβ40 as well as the extracellular makers fell over time in whole brain and in the CSF secreting CP (Fig 1,2). These generated linear relation curves, by which the half time for efflux was calculated as in the Table 1. Table 1 showed the efflux half time of both ^{125}I-T_4 and ^{125}I-Aβ40 were shorter than their size matched extracellular markers (mannitol and inulin), suggesting specific transporters for ^{125}I-T_4 and ^{125}I-Aβ40.

2.3. Effects of TTR on ^{125}I-T$_4$ / ^{125}I-Aβ40 in the brain

In presence of TTR, levels of ^{125}I-T$_4$ fell over the time but at slower slopes, leading to significant prolonged half time of ^{125}I-T$_4$ efflux in brain parenchyma (Fig 1, Table 1). However, TTR did not significantly change the ^{125}I-Aβ40 efflux half time in brain parenchyma (Fig 2, Table 1). There was a noticeable increase in the uptake of ^{125}I-T$_4$ and ^{125}I-Aβ40 in the CP in presence of TTR compared to the baseline, whilst uptakes of ^{3}H-mannitol and ^{3}H-inulin remained similar to control experiments (Fig 3,4). TTR had no significant effect on the integrities of ^{125}I-Aβ40, as the percentage of loss of ^{125}I activity (cpm in supernatant) were almost equal in treatment groups (41.3±4.5% and 42.7±5% in TTR 50, 200 μg.ml^{-1} respectively, versus 41.5±6.3% in control group, $p>0.05$).

3. DISCUSSION

This study investigated the effect of TTR on T$_4$ and amyloid efflux from the CSF. At baseline (without TTR), both ^{125}I-T$_4$ (775 Da) and ^{125}I-Aβ40 (4455 Da) were cleared from brain faster than their corresponding extracellular markers, ^{3}H-mannitol (182 Da) and ^{3}H-inulin (5000 Da), consistent with the concept of specific transporters for ^{125}I-T$_4$ and ^{125}I-Aβ40, in addition to the diffusion and fluid drainage pathways accessible by mannitol and inulin. With TTR in aCSF, the loss of thyroxin from the brain was significantly reduced (i.e. efflux half time was prolonged), while the retention of Aβ40 in the brain was not significantly affected (i.e. efflux half time was not changed). In presence of TTR, the accumulation of both ^{125}I- T$_4$ and ^{125}I-Aβ40 in the CP were significantly increased. Since the nascent CSF is washed out from the ventricular system during perfusion with artificial CSF in the experiments, the CSF endogenous TTR approaches zero in these mice during the perfusion.

TTR is a protein that binds and distributes the TH in the body. In the blood, >99% of TH is bound to specific plasma proteins known as TH distributor proteins, such as TBG, TTR, albumin and ApoB100 [7]. These TH binding proteins maintain the large extrathyroidal pool of TH which can be liberated with great rapidity for entry into cells, ensure a constant supply of TH to the cells and tissues [1, 7]. In the CSF, TTR assumes the main role in terms of TH homeostasis, as TTR has higher affinity to T$_4$ than the albumin (ca 5-9 x 10^{-8}M vs ca 7 x 10^{-5}M) [1]. We showed here that TTR affected T$_4$ distribution in the brain and prevented the T$_4$ loss from

the brain. The increased accumulation of T_4 in the CP by TTR may be due to reduced basolateral efflux (from choroid epithelial cells to plasma) as well as an augmented uptake of T_4 from CSF. The T_4 efflux from the CSF involves a number of transporters [29] and is saturable in presence of unlabelled T_4 [9, 11, 30]. Despite the important role of TTR in TH homeostasis, mice lacking the TTR gene maintain normal functioning of thyroid glands in the face of significant reduction in total serum T_4 concentration [9]. Using the same model it was found that TTR was not essential for TH to reach the brain [8]. This is not surprise given there are numerous mechanisms for T_4 transport, and a number of thyroxine binding proteins may take over the role of TTR in TTR-null mice. Despite the apparent normal distribution of T_4 in the brain, TTR knock-out mice have behavioural changes, which are associated with an apparent increase of noradrenaline in the limbic forebrain of the mice [31, 32].

It has been suggested that TTR has other important physiological effects. TTR could bind Aβ and prevent amyloid formation, and also inhibit Aβ induced cytotoxicity [13, 14, 18].

Aβ deposition in the brain has been linked to the AD pathogenesis. The accumulation of Aβ in the AD brain is suggested to be related to defective Aβ clearance from the brain rather than increased Aβ production [33]. P-gp and low-density lipoprotein receptor-related protein-1 (LRP-1) in cerebrovasculature have been reported to play important roles in Aβ clearance from the brain to the blood [34]. A recent study demonstrated that TTR interacted with Aβ directly and stimulated brain-to-blood but not blood-to-brain Aβ permeability in hCMEC/D3, a human cerebral microvascular endothelial cell line [35]. Together with one of their previous studies [36], they conclude that TTR acts as an Aβ carrier that transports the peptide to its receptor both at blood brain barriers in the brain and at the liver in the peripheral system [35, 36]. In our study, we intended to use TTR concentrations close to the physiology level in mouse CSF (20-50 μg/ml) [37], which are more than that of ^{125}I –Aβ40. The TTR dose effect was not seen. Thus, we could use one dose of TTR (i.e.50 μg/ml) in the study. That will be close to the clinical setting. At physiological condition in human, TTR is abundant (5-20 μg/ml or 0.1-0.36 μM) [38] while Aβ concentration in CSF is relatively low (3nM or less) [39, 40]. The effects of TTR on Aβ neurotoxicity have also been demonstrated in *in vivo* experiments. The silencing the endogenous TTR gene accelerated the appearance of Aβ associated neuropathology, while genetically programmed overexpression of a human TTR transgene suppressed both the neuropathologic and behavioural abnormalities in the APP23 transgenic mouse model [41]. Backcrossing of the TTR null mice with an AD mouse model resulted in an increase in the deposition of Aβ in the brains of mice with the hemizygous deletion of TTR [42]. TTR concentration in the CSF was found to negatively correlate with AD patients [43], as well as the degree of dementia in patients with AD [44]. However, TTR

was not found to change in AD patients compared to age-matched controls in a number of larger AD clinical studies such as ADNI (The Alzheimer's disease neuroimaging initiative) [28, 45].

As a main tissue in the brain to secrete CSF and synthesize TTR, the CP is suggested to be a major site of Aβ removal in the brain. In a triple transgenic mouse model of AD, CP dysfunction was shown linking to increasing Aβ burden resulting in reduced production of CSF and diminished secretion of TTR [46]. In AD patients, CSF turnover / clearance is decreased by 50% [47]. It will decrease Aβ40 clearance from the brain and increase the contact time for proteins with glucose in the CSF, which promotes the glycosylation of CSF proteins and increased oxidative stress [48]. The CP also contains high levels of Aβ degrading enzymes, such as neprilysin, endothelin-converting enzyme-1, insulin degrading enzyme [19], which degrade β-amyloid peptides. In our study we showed TTR bound to Aβ40 and distributed Aβ40 to the CP, as the CP has high affinity to TTR due to presence of TTR receptor [49]. We found significant loss of the integrity of ^{125}I-Aβ / TTR complex in the brain tissue detected by the TCA precipitation assay. The loss of radioisotope activity in the precipitate could be due to involvement of enzymatic degradation in the CP, blood brain barrier or brain parenchyma. The accumulation / degradation of Aβ40 in the CP enhance Aβ40 removal which is known to occur at the CPs via receptor mediated processes [e.g. via the LRP1] that are modulated by chaperone complexes such as ApoE, clusterin, TTR [50]. In the CP, the abundance of LRP-1 increases with age, whereas the abundance of LRP-2 (megalin) is reduced during normal ageing in rodent [51]. Megalin is thought to contribute to the clearance of Aβ through the CP [52], and patients with AD have reduced levels of megalin at the CP [53]. Since the BBB is not intact with the clearance deficits in the AD patients, the role of Aβ transport and degradation at the CP becomes increasingly more prominent and needs further investigating.

In conclusion, this study demonstrates that TTR acts differentially on T_4 and Aβ40 efflux from the CSF. TTR prevents the loss of thyroxine from the CSF, a positive adaptation since T_4 is a substrate for multiple efflux transporters from the CSF, but is a necessary requirement for normal brain function. TTR does not significantly affect the retention of Aβ40 in the brain parenchyma, but redistributes Aβ40 to the CP for degradation and clearance. As a major site of TTR synthesis in the brain, the CP is an important site for homeostasis of TTR binding hormone and peptides.

4. METHODS

4.1. Mouse lateral ventricular perfusion

All procedures were within the guidelines of the Animals (Scientific procedures) Act, UK, 1986. Eight week old C57/BL6 male mice (20-25g) were ordered from Harlan (UK) and were kept on a 12-h light/dark cycle with food and water freely available. They were anaesthetized with i.p. 0.1 ml Vetalar (100mg.ml^{-1} ketamine anaesthetic) and 0.2 ml Domitor (1mg.ml^{-1} medetomidine analgesic/sedative) and injected with heparin (1000 U.Kg^{-1}). The mice were put on a stereotaxic frame, the scalp removed and a hole made in the skull above each lateral ventricle, 1.0 mm lateral and 1.0 mm posterior to the bregma. A 33 gauge needle was used to make the hole, and had a tubing guard which kept the depth of the holes constant (2.5 mm).

A syringe driver contained artificial CSF (aCSF) (NaCl: 6.821; KCl: 0.2238; $MgCl_2$ $6H_2O$: 0.1626; Na_2HPO_4: 0.0994; $NaHCO_3$: 1.512; Urea: 0.1202; Glucose: 2.178; $CaCl_2 4H_2O$: 0.2058; g.L^{-1}) with either ^{125}I-T_4 (0.037 MBq.ml^{-1}; 8.62 nM) plus ^{3}H-mannitol (0.148 MBq.ml^{-1}; 26.47 µM) or ^{125}I-Aβ40 (0.09 MBq.ml^{-1}; 1.1 nM) plus ^{3}H inulin (0.37 MBq.ml^{-1}; 15.4 µM) was run at 1µl.min^{-1} for 1 min at either absence or presence of TTR (50 or 200 µg.ml^{-1}). Aβ40 was studied in this study, as it is several folds higher than Aβ42 in the brain and its accumulation disrupts the integrity of BBB [54], although Aβ40 is less pathogenic than Aβ42 due to its low propensity to aggregate [55]. The radio-labelled mannitol (182 Da) and inulin (5000 Da) acted as size-matched, non-transported, extracellular markers for ^{125}I-T_4 (775 Da) and ^{125}I-Aβ40 (4455 Da) respectively. Radio-isotopes were purchased from GE Life Sciences (Buckinghamshire, England) and Perkin-Elmer (Boston, USA).

Human TTR (huTTR) was obtained from Biodesign International (Maine, USA) and dissolved in saline. 100 µl of either ^{125}I-T_4 (0.037 MBq.ml^{-1}; 8.62 nM) or ^{125}I-Aβ40 (0.09 MBq.ml^{-1}; 1.1 nM) was mixed with 1 ml of TTR (50 / 200 µg.ml^{-1}; 0.909/3.636 µM). The mixture was allowed to bind for 30 min at room temperature and was then dialyzed overnight at 4 °C in seamless cellulose tubing (with a cutoff of 12kD mol wet) (St. Louis, MO, USA) to remove any unbound T_4 or Aβ40.

4.2. Sampling of the brain tissue, TCA precipitation and radioactive counting

At 2, 4, 8, 16, 24 minutes after the injection, the mice were killed with overdose sodium pentobarbital. The heart was exposed and blood taken using a syringe inserted in the left ventricle. The brain was then washed with cold PBS and was dissected into right and left frontal cortex (FX), choroid plexus (CP), ependymal lining lateral ventricles (EP), hippocampus (HC), cerebellum (CB) and rest of brain (ROB). Samples were weighed on a Cahn microbalance (Thermo, USA), and homogenized with PBS in a glass homogenizer.

The TCA precipitation method was used to determine how much ^{125}I was still attached to Aβ at the end of the experiments [56]. To measure intact ^{125}I-Aβ, one volume of TCA (20%) was added to the sample, and then samples were vortexed, incubated in ice for 30 min and centrifuged at 14,000 rpm at 4 °C for 30 min. Following centrifugation, radioactivity of precipitated ^{125}I-Aβ40 (intact peptide) and TCA supernatant (degraded peptide) were measured on a LKB Rackbeta liquid scintillation counter (Wallac). Solvables (National Diagnostic, UK) was added to each of samples to dissolve organic samples overnight. The radioactivities of the tissues and 100 μl of infused aCSF were determined by liquid scintillation counting (LKB Wallac Rackbeta Spectral 1219 counter) after the addition of 3.0 ml liquid scintillation fluid (Ultima Gold, Packard Bioscience, Netherland).

4.3. Expression of results

Regional brain uptake (volume of distribution) was calculated using equation:

$$Vd\ (ml.g^{-1}) = 100*(Tissue\ dpm.g^{-1}) / (aCSF\ dpm.ml^{-1}) \quad (1)$$

The half-time for efflux of ^{125}I-T_4 and ^{125}I-Aβ40 from brain was calculated according to Banks et al [57]. Briefly, LogVd was plotted against time and the slope of the line was calculated where:

Efflux half time (min) = Log2 / slope of line (2)

4.4. Data analysis

All values were expressed as mean SEM. ANOVA and unpaired t-test as appropriate were used to compare means. Values of $p<0.05$ were considered statistically significant.

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FIGURE LEGENDS

Figure 1. ^{125}I-T_4 efflux after lateral ventricle injection. Levels of ^{125}I-T_4 and its size matched extracellular marker ^{3}H-mannitol fell over time in whole brain. ^{125}I-T_4 was cleared faster than ^{3}H-mannitol at the baseline (A). The addition of TTR (B: 50 μg.ml^{-1}; C: 200 μg.ml^{-1}) slowed the ^{125}I-T_4 clearance but did not affected ^{3}H mannitol one. Solid squares are ^{125}I-T_4 LogVd while open squares represent ^{3}H-mannitol LogVd. Values are mean ± SEM, n=3-5.

Figure 2. ^{125}I-Aβ40 efflux after lateral ventricle injection. Levels of ^{125}I-Aβ40 and its size matched extracellular marker ^{3}H-inulin fell over time in whole brain. ^{125}I-Aβ40 was cleared faster than ^{3}H-inulin at the baseline (A). The addition of TTR (B: 50 μg.ml^{-1}; C: 200 μg.ml^{-1}) did not affect the ^{125}I-Aβ40 and ^{3}H-inulin clearance (B, C). Solid circles are ^{125}I-Aβ40 LogVd, while open circles represent ^{3}H-inulin LogVd. Values are mean ± SEM, n=3-5.

Figure 3. Regional brain uptake of ^{125}I-T_4 at 16min post lateral ventricle injection. Baseline uptake of ^{125}I-T_4 showed highest accumulation in the CP (choroid plexus). The ependymal ventricular lining (EP) also showed some uptake of ^{125}I-T_4, however, the rest of brain did not show much uptake. The addition of TTR to the lateral ventricles resulted in increased retention of ^{125}I-T_4 in the CP, and the increase reached significance with TTR 200 μg.ml^{-1}.

The addition of TTR did not change retention of ^{125}I-T_4 in EP, HC, FX and CB. HC = hippocampus, FX = front cortex, CB = cerebellum. Values are mean ± SEM, n=3-5. * means $p<0.05$ compared to baseline.

Figure 4. Regional brain uptake of ^{125}I-Aβ40 at 16min post lateral ventricle injection. Baseline uptake of ^{125}I-Aβ40 showed highest accumulation in the CP (choroid plexus). Brain parenchymal uptake was lower, and only the ependymal ventricular lining (EP) showed some uptake of ^{125}I-Aβ40. The addition of TTR to the lateral ventricles resulted in significant increased retention of ^{125}I-Aβ40 in the CP, but did not change retention of ^{125}I -Aβ40 in EP, HC, FX and CB. HC = hippocampus, FX = front cortex, CB = cerebellum. Values are mean ± SEM, n=3-5. * means $p<0.05$ compared to baseline.

Table 1. Efflux half time of ^{125}I-T_4, ^{125}I-Aβ and the extracellular markers ^{3}H-mannitol, ^{3}H-inulin in whole mouse brain in absence and presence of TTR

Efflux half time (min) in whole brain	^{125}I-T_4	^{3}H-mannitol	^{125}I-Aβ	^{3}H-inulin
Baseline (n=5)	5.16 ± 0.27	7.44 ± 0.26	8.34 ± 0.39	10.78 ± 0.7
TTR 50 μg.ml^{-1} (n=4)	7.88 ± 0.35*	7.74 ± 0.25	9.04 ± 0.17	11.32 ± 0.62
TTR 200 μg.ml^{-1} (n=3)	7.97 ± 0.31*	7.92 ± 0.40	9.23 ± 0.22	10.68 ± 0.80