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Alpha Synuclein is Transported Into and Out of the Brain by the Blood-brain Barrier

Yu-Ting Sui¹, Kristin M. Bullock², Michelle A. Erickson², Jing Zhang¹, and W. A. Banks^{2,3}

¹Department of Pathology, University of Washington School of Medicine, 325 9th Ave, HMC Box359635, Seattle WA 98104

²Geriatrics Research Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Room 810 Bldg 1, 1660 S. Columbian Way, Seattle, WA 98108

³Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Washington School of Medicine

Abstract

Alpha-synuclein (α -Syn), a small protein with multiple physiological and pathological functions, is one of the dominant proteins found in Lewy Bodies, a pathological hallmark of Lewy body disorders, including Parkinson's disease (PD). More recently, α -Syn has been found in body fluids, including blood and cerebrospinal fluid, and is likely produced by both peripheral tissues and the central nervous system. Exchange of α -Syn between the brain and peripheral tissues could have important pathophysiologic and therapeutic implications. However, little is known about the ability of α -Syn to cross the blood-brain barrier (BBB). Here, we found that radioactively labeled α -Syn crossed the BBB in both the brain-to-blood and blood-to-brain directions at rates consistent with saturable mechanisms. Low-density lipoprotein receptor-related protein-1 (LRP-1), but not p-glycoprotein, may be involved in α -Syn efflux process, and lipopolysaccharide (LPS)-induced inflammation could increase α -Syn uptake by the brain by disrupting the BBB.

Keywords

Blood-brain barrier; Alpha-synuclein; LRP-1; p-glycoprotein; Parkinson's disease

1.0 Introduction

Parkinson's disease (PD) is among the most common neurodegenerative disorders affecting 1–2% of the ageing population [56]. Pathologically, the disease involves multiple brain regions, including progressive loss of dopaminergic (DA) neurons, particularly in the substantia nigra pars compacta (SNpc) and in the locus ceruleus [27]. The histological

Corresponding author: WA Banks, wabanks1@uw.edu; phone: 206 764 2701; Geriatrics Research Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Room 810 Bldg 1, 1660 S. Columbian Way, Seattle, WA 98108.

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hallmark of PD is the presence of inclusion body formation called Lewy bodies (LBs) in remaining neurons.

Alpha-synuclein (α -Syn) is the major component of LBs, and accumulations of α -Syn aggregates are also found in dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [54]. Physiologically, α -Syn in the brain at multiple cellular locations, including presynaptic terminals. Substantial evidence has shown association between α -Syn and familial and sporadic PD. For example, mutations in the α -Syn gene (SNCA) can cause familial PD [35, 49, 62] and multiplication of the normal wild-type SNCA can also increase the risk of sporadic PD [16, 28, 43, 52].

Although α -Syn is considered an intracellular protein, a large body of literature has found the presence of α -Syn in biological fluids including human cerebrospinal fluid (CSF) and blood plasma [12, 23, 38, 40, 45, 58]. Most studies showed decreased CSF levels of total α -Syn in patients with PD compared to controls [30, 44, 58], whereas the levels of oligomeric forms were increased in PD patients compared to controls [24, 45, 57]. Defective transport mechanisms of α -Syn from parenchyma to the CSF may account for the elevated load of α -Syn. However, little is known about the mechanisms responsible for α -Syn transportation.

The blood-brain-barrier (BBB) is an important regulator of the CNS environment, controlling the exchange of substances between the central nervous system (CNS) and the peripheral circulation, determining the concentration of molecules in the CNS, and contributing to the normal function of the brain. The vascular BBB is formed by brain endothelial cells that are joined by tight junctions, giving the BBB the ability to transport ions, peptides, and regulatory proteins selectively between the brain and the periphery [14]. Recently, evidence has shown that a dysfunctional BBB may play a role in the pathogenesis and progression in PD [1, 34, 37, 46]. Altered transport mechanisms, such as defective expression of efflux pumps, altered transporter affinities, and receptor dysfunctions are all potential pathologies of the BBB in PD. By analogy, defective efflux of amyloid beta protein (A β) by low-density lipoprotein receptor-related protein-1 (LRP-1) and P-glycoprotein occurs with both Alzheimer's disease and with inflammation and is thought to be a major factor in the accumulation of A β by brain [25].

Levels of α -Syn in brain and blood are likely responsive to disease onset and progression. A mechanism that has not been understood is the possibility that α -Syn could cross the BBB. In this study, we examined the transport of α -Syn across the BBB in both the blood-to-brain and brain-to-blood directions by injecting radioactively labeled α -Syn into mice and investigating the roles of LRP-1 and P-glycoprotein in its transport.

2.0 Material and Methods

2.1 Animal Use

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) and performed at a facility that is approved by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). CD-1 male mice (8

weeks old) were used for all studies (Charles River, Wilmington, MA). Mice were given ad lib access to food and water and kept on a 12/12 hour light/dark cycle until study.

2.2 Radioactive labeling

α -Syn (rPeptide, Athens, GA) and murine amyloid beta peptide₁₋₄₂ (A β ₁₋₄₂) (Bachem) were radioactively labeled with Na ¹²⁵I (Perkin Elmer, Waltham, MA) by the chloramine-T (Sigma-Aldrich, St. Louis, MO) method. Albumin (Sigma-Aldrich, St. Louis, MO) was radioactively labeled with Na¹³¹I (Perkin Elmer) by the chloramine-T method. Radioactively labeled α -Syn (I-Syn), radioactively labeled A β ₁₋₄₂ (I-A β), and radioactively labeled albumin (I-Alb) were purified on a column of Sephadex G-10 (Sigma-Aldrich, St. Louis, MO). The I-Syn eluted as a single peak with a molecular weight of 20,000 by autoradiographic gel immediately, 72h, and 11 days after labeling with greater than 90% of the radioactivity precipitating with acid.

2.3 Preparation and administration of lipopolysaccharide (LPS)

LPS from *Salmonella typhimurium* (Sigma) was dissolved in 0.9% NaCl and 3 mg/kg given in a volume of 0.2 ml by intraperitoneal (ip) injections at t = 0, t = 6 h, t = 24h. Mice were studied at 28 h after the first injection.

2.4 Efflux-Competition

An ICV method was used to assess brain-to-blood efflux rates [4, 6]. Mice were anesthetized with an ip injection of 0.15ml of 40% urethane (Sigma-Aldrich, St. Louis, MO). The scalp was removed and a hole was made into the skull (0.5mm posterior and 1mm lateral to the bregma) and 1 μ l of lactated Ringers solution containing 2.5 $\times$ 10⁴ CPM of I-Syn was injected into the lateral ventricle of the brain (icv) using a 1.0 μ l Hamilton syringe. For efflux kinetics studies, mice were decapitated at 2, 5, 10, 20, or 60 min. For self-inhibition studies, 2.5 $\times$ 10⁴ CPM of I-Syn with or without 1 μ g of unlabeled α -Syn was injected icv and mice were decapitated 5 minutes after icv injection. Other mice received an injection of I-Syn with or without 1 μ g of unlabeled murine A β ₁₋₄₂. The whole brain was removed and weighed. The level of I-Syn available for transport at t = 0 was estimated by repeating this procedure in mice that had been overdosed with urethane and had been dead for 10–20 min [3, 5]. The levels of radioactivity in the brain samples were counted for 3 minutes in a gamma counter and divided by brain weight in grams to yield cpm/g. The amount of I-Syn that was transported out of the brain (%T) was determined with the equation:

$$\%T = [100(\text{cpm/g})_0 - (\text{cpm/g})_5] / (\text{cpm/g})_0$$

where (cpm/g)₀ is the mean level of cpm/g for the t=0 group and (cpm/g)₅ is the individual mouse's level of cpm/g at t = 5 min.

Other mice were prepared as above but received an icv injection of I-A β with or without 1 μ g unlabeled α -Syn. Because of the slower efflux rate of I-A β , these mice were studied 10 min rather than 5 min after receiving the icv injection.

2.5 Brain Perfusion

A brain perfusion method [2] was used to assess whether I-Syn was a substrate of the p-glycoprotein efflux transporter. Mice were anesthetized with an I.P. injection of urethane (40%). The heart was exposed, by opening up the thorax. The descending aorta was clamped and both jugular veins were severed. A 23-gauge butterfly needle was injected into the left ventricle of the heart. Zlokovic buffer (7.19g/l NaCl, 0.3 g/l KCl, 0.28 g/l CaCl₂, 2.1 g/l NaHCO₃, 0.16 g/l KH₂PO₄, 0.17 g/l anhydrous MgCl₂, 0.99 g/l D-glucose, and 10 g/l bovine serum albumin added on the day of perfusion) containing 200,000 cpm/ml I-Syn and 200,000 cpm/ml I-Alb with or without 10ug/mL cyclosporine was infused at a rate of 2 ml/min. After 2 minutes of perfusion, the mouse was decapitated and the brain was removed and weighed. The brain was then placed into a gamma counter for 3 minutes to determine the levels of radioactivity. Brain/perfusion ratios were calculated, expressed as $\mu\text{l/g}$, and the ratios for I-Syn corrected for vascular space by subtracting the ratios for I-Alb.

2.6 Influx

Multiple-time regression analysis was used to measure blood-to-brain uptake of I-Syn [11, 47]. Mice were anesthetized with an I.P. of urethane (40%) and a 200 μl injection of lactated Ringers solution containing 3×10^5 cpm I-Syn was injected into the jugular vein. Between 1 to 10 minutes after the injection, arterial blood was collected from the carotid artery and the brain was removed and weighed. Levels of radioactivity in the brain and 50 μl of serum were counted in a gamma counter for 3 minutes. Brain/serum ratios were calculated to yield units of $\mu\text{l/g}$ and plotted against exposure time. The linear portion of the slope of the resulting correlation measures the unidirectional influx rate in units of $\mu\text{l/g-min}$ and the Y-intercept measures the distribution space in brain at $t = 0$ in units of $\mu\text{l/g}$.

2.8 Statistical Analysis

Regression analysis and other statistical analyses were performed with the use of Prism 5.0 (GraphPad Software Inc., San Diego, CA). Means are reported with their SE and compared by one way analysis of variance (ANOVA) followed by Newman-Keuls post-test; t-test was used only in studies that compared two means. Slopes of the lines were compared with the regression package contained in Prism 5.0.

2.9. In Vivo Stability of I-Syn in Brain and Blood

Arterial serum and whole brain were obtained 10 or 60 min after the iv injection or 2 and 10 min after ICV injection of I-Syn. Blood was allowed to clot, was centrifuged at 5400 rpm for 15 min, and 50 microl of the resulting serum added to 500 microl of lactated Ringer's solution containing 1% BSA (LR-BSA), this mixture mixed, 500 microl of 30% trichloroacetic acid (TCA) added, mixed, and this mixture centrifuged for 15 min at 5400 rpm. Resulting supernatant and precipitate were counted separately and the percent of counts in the precipitate was calculated. Hemi-brains were homogenized in 0.5 ml of LR-BSA with a bead beater for 30 sec at 4800 rpm x 2 on ice. Samples were transferred to a 12x75 mm glass tube and centrifuged at 5400 rpm for 15 min and a portion of the resulting supernatant added to an equal volume of 30% TCA, mixed, and centrifuged at 5400 rpm for 15 min. Resulting supernatant and precipitate were counted separately and the percent of counts in

the precipitate was calculated. To correct for any degradation that might have occurred during the processing for acid precipitation, we added I-Syn to non-radioactive arterial whole blood or to whole brain and processed as above. Biological samples were corrected for degradation during processing by dividing their values by the processing control values.

3.0 Results

Efflux of I-Syn from brain after ICV injection showed a biphasic curve with an early phase half-life of 1.4 min and a late phase half-life of 23.5 min (Figure 1). Table I shows that the radioactivity in brain and blood after ICV injection was mostly precipitated by acid. Neither one microg of unlabeled α -Syn (Figure 2, upper left panel) nor of A β ₁₋₄₂ (Figure 2, upper right panel) inhibited the efflux of I-Syn. However, one microg of unlabeled α -Syn significantly inhibited I-A β efflux (Figure 2, lower panel).

I-Syn was taken up by brain with brain perfusion, but this uptake was not affected by the P-glycoprotein inhibitor cyclosporine. Figure 3 shows brain/perfusion ratios after correction for the vascular space as measured with I-Alb.

I-Syn crossed the BBB in the blood-to-brain direction after its IV injection with a unidirectional influx rate of 0.54 ± 0.20 microl/g-min (Figure 4). Table I shows the radioactivity in brain and blood after IV injection was mostly precipitated by acid. LPS disrupted the BBB to I-Alb, increasing the I-Alb space in brain from 9.3 ± 0.3 microl/g of brain to 15.4 ± 0.45 microl/g of brain. LPS also increased the Y-intercept for I-Syn as shown in figure 4 but did not affect the slope as compared statistically with the Prism 5.0 program.

4.0 Discussion

Our findings demonstrate that α -Syn is transported across the BBB bidirectionally; that is, in both the brain-to-blood and blood-to-brain directions. Figure 1 shows a very rapid efflux rate of under 2 minutes, making this the most rapidly effluxed protein found to date. The efflux half-life of murine A β ₁₋₄₂, for example, is about 16 min [10]. Such a rapid efflux rate is consistent with a saturable transport system such as LRP-1 or p-glycoprotein, the efflux transporters for A β [17, 20, 21, 29]. Consistent with this is the ability of unlabeled α -Syn to inhibit I-A β efflux; this suggests that LRP-1 is likely involved in I-Syn efflux from brain. Intriguingly, I-Syn efflux was not inhibited either by unlabeled α -Syn or murine A β ₁₋₄₂, the latter given at a dose that readily inhibits I-A β efflux. This contradiction of rapid efflux rate and lack of inhibition may be explained if the transporter has a high capacity for I-Syn efflux. Additionally, the involvement of exosomes in α -Syn efflux as recently demonstrated by us may also change saturation pharmacokinetics [51].

The inability of cyclosporine to inhibit I-Syn uptake suggests that p-glycoprotein, the other efflux system of A β ₄₂, is not involved in I-Syn efflux. P-glycoprotein is located on the blood-side of the BBB and it prevents its ligands that would otherwise cross the BBB from being taken up from the blood. As such, the brain perfusion method used here is a reliable method for assessment with inhibitors included in the perfusion buffer increasing brain uptake of p-glycoprotein substrates.

The brain perfusion experiment also showed that I-Syn can cross the BBB. We showed I-Syn crossed the BBB after intravenous administration as well, using multiple-time regression analysis to measure brain uptake. The unidirectional influx rate of about 0.54 microl/g-min is similar to that measured for leptin and interleukin-1 [7, 8], two proteins of similar size that are known to have effects on brain that depend on their crossing the BBB. LPS, a derivative of the cell wall of Gram negative bacteria that is a potent inducer of the innate immune system, alters the BBB transport of A β . Here, LPS likely increased the blood-to-brain uptake of I-Syn, but by the mechanism of disrupting the BBB rather than by increasing its transporter.

The bidirectional transport of α -Syn suggests that blood-borne α -Syn could contribute to brain levels of α -Syn, that efflux from brain could contribute to blood levels, and that efflux could be an important mechanism by which α -Syn levels in brain are regulated. Acid precipitation was used to show that the radioactivity in serum and brain homogenates was not free, but still attached to its protein. Two other proteins, A β and cellular prion protein, also show bidirectional transport between CNS and peripheral tissues [9, 22], and both are considered toxic to the CNS if misfolded [18, 53]. In the case of A β whose concentration is much higher in the CSF than the blood, impairment in its efflux has been shown to be a major contributor to its accumulation in brain. It is unclear what role the BBB transporters play in accumulation of α -Syn in the PD brain. This line of research could be quite informative because recent results have suggested that the BBB might be altered in PD [31, 41]. Therefore, at least in theory, defective α -Syn transport by the BBB can increase accumulation of α -Syn in CNS. Our results indicated LRP-1 is a potential efflux transporter of α -Syn. Recently, increased expression and redistribution of LRP-1 in melanized neurons was observed in patients with PD [59], suggesting that alteration in α -Syn transport might contribute to PD pathogenesis.

Alternatively, our results are consistent with the supposition that blood levels of α -Syn could contribute to CNS pathology, especially as plasma levels of α -Syn are hundreds of times higher than its CSF levels [51]. To this end, α -Syn has been shown to be a potentially toxic protein to neurons, and plays a causal role in PD [19]. Delivering α -Syn directly to the SN with viral vectors in mice [55], rats [36, 42, 60], or primates [26, 32, 61] can cause significant neuron loss and α -Syn deposition.

BBB transport can be altered in pathological conditions, including neuroinflammation. Chronic microglial activation, as well as dopaminergic neurodegeneration, have been observed after LPS injection [15, 50]. Systemic inflammation has been suggested to have effects on immune cells in the brain, and results in chronic neurodegenerative disorders such as PD [48]. This correlates with our findings that LPS increased blood-to-brain uptake of α -Syn. Recent studies showed rat brain oligodendrocytes [33], human primary astrocytes, microglia, and cortical neurons [13] are able to take up α -Syn from the environment *in vitro*, and the cells exposed with α -Syn demonstrated changes in the gene expression profile, with up-regulation of pro-inflammatory cytokines and chemokine [39].

In conclusion, α -Syn is transported across the BBB bidirectionally, in both the brain-to-blood and blood-to-brain directions. Our results are consistent with efflux being mediated in

part by LRP-1 but not p-glycoprotein. Inflammation likely enhances the blood-to-brain uptake of α -Syn by the mechanism of disrupting the BBB, leading to enhanced neurotoxicity.

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Abbreviations

α-Syn	Alpha-synuclein
Aβ	Amyloid-beta Protein
BBB	Blood-brain Barrier
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DA	Dopaminergic
ICV	Intracerebroventricular
LBs	Lewy Bodies
LPS	Lipopolysaccharide
LRP-1	Low-density Lipoprotein Receptor-related Protein-1
PD	Parkinson's Disease
SN	Substantia Nigra

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Highlights

- Alpha-synuclein crosses the blood-brain barrier in both the blood-to-brain and brain-to-blood direction
- Inflammation as induced by Lipopolysaccharide increases alpha-synuclein entry into brain, probably because of BBB disruption

Figure 1.

Clearance from brain of alpha synuclein radioactively labeled with ^{125}I (I-Syn). The I-Syn was injected into the lateral ventricle of the brain and the residual counts over time determined. Clearance was biphasic with an early, rapid phase of 1.4 min and a later, slower phase of 23.5 min.

Figure 2.

Inhibition of I-Syn efflux. Efflux of I-Syn was not inhibited by unlabeled alpha-synuclein nor by unlabeled A β . However, radioactively labeled A β was inhibited by unlabeled alpha synuclein.

Figure 3.
P-glycoprotein and I-Syn. Efflux of I-Syn was not inhibited by the p-glycoprotein substrate cyclosporine. The brain perfusion method was used.

Figure 4.

Blood-to-brain Influx of I-Syn. Multiple-time regression analysis was used to measure the influx of I-Syn into brain. The entry rate of untreated (NS) mice was 0.54 ± 0.20 $\mu\text{mol/g-min}$. Treatment with LPS did not increase the rate of entry but did increase the Y-intercept value, suggesting enhanced entry into brain because of BBB disruption.

Table I

Percent Acid Precipitation of Radioactivity Found in Brain and Blood after ICV or IV Injection of I-Syn.

	Injection Method			
	Intravenous		Intracerebroventricular	
	10 min [*]	60 min [*]	2 min [*]	10 min [*]
Serum	99.8 +/- 0.03	92.9 +/- 1.3	75.5 +/- 5.3	80.4 +/- 5.9
Brain	98.7 +/- 0.01	79.7 +/- 1.8	98.0 +/- 0.8	100 +/- 0.5

* Time from injection to harvesting. Mean +/- SE reported (n = 2/cell)