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Evaluation of mechanisms for accessing intracellular targets for protein-based drugs

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Abstract

Over the years, biological drugs have evolved and have made breakthroughs in diseases associated with extracellular proteins. However, intracellular proteins that cause disease progression are still largely inaccessible. Examples of diseases that are caused by an intracellular aggregation of proteins are neurodegenerative diseases such as Parkinson's disease, Huntington's disease (HD), and Alzheimer's disease (AD).

The purpose of the work is to find a strategy to reach the neurons intracellularly. The goal is to be able to design a biological drug that enters the neuron by investigating different uptake mechanisms. A systematic review of 43 published studies was reviewed, and the results could be obtained. All result presents data from different receptors, cell-penetrating peptides, and adeno-associated viruses (AAV) that were examined. It showed that there are advantages and disadvantages with all the uptake mechanisms. There are risks of side effects for each uptake mechanism, and further studies are required to consider the risk. AAV2 and the neuron-specific receptors lack specific information about their mechanism, but there is a high potential to develop these strategies. Both AVV and the neuron-specific receptors provide specific uptake into tissues.

Keywords: intracellular target, neuronal uptake, biological drug, receptor mediated uptake, cell-penetrating peptides.

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1. Introduction

A *biological drug* is defined as the active substance is produced or purified from a biological origin, such as living cells. Unlike ordinary drugs, biological drugs are much larger and more complex [1].

The development of biological drugs has, over the years, made breakthroughs in diseases associated with extracellular proteins. However, intracellular targets are still challenged in today's research, and two-thirds of the world's diseases have their pathogenic aggregate inside the cell [2].

An example of such a disease is Parkinson's disease (PD). It is the second most common neurodegenerative disease, affecting 2-3% of the population over 65 [3]. The disease is characterized by tremor, bradykinesia, and rigidity [4]. The pathophysiology behind the disease is currently unclear, as PD is a multifactorial disease [3]. Published studies have shown that, among other factors, the aggregated form of alpha-synuclein proves to cause the disease [5, 6]. Alpha-synuclein, a constituent of Lewy-bodies, begins its aggregation intracellularly, like other proteins such as huntingtin (Htt) and tau [7]. When these aggregates spread in the brain, it leads to a cognitive decline. An accumulation of alpha-synuclein in the substantia nigra worsens the course of the disease [8]. It decreases dopaminergic neurons, and the dopamine supply to the basal ganglia decreases [4].

There are currently no cures for PD, and today's medication is not sustainable in the long term [4]. Today's treatment for PD involves an increased level of dopamine. However, it does not slow down the progression of the disease, and the drug's effect decreases. Today, L-dopa is a first-hand drug, and with long-term use, it can lead to several disabilities [4].

1.1 Intracellular targets

In addition to Parkinson's disease, other neurodegenerative diseases that are caused by intracellular aggregation of proteins, are Huntington's disease (HD) and Alzheimer's disease (AD). AD is caused, among other factors, by an aggregation of tau, and HD is caused by the huntingtin protein (Htt) [9, 10]. For Parkinson's disease, alpha-synuclein is a possible therapeutic target. Alpha-synuclein consists of 40 amino acids and is encoded by the SNCA gene in a human cell. Alpha-synuclein is found mainly in the neuronal presynaptic terminals of the brain [3]. In neurons, these play a significant role in releasing several transmitters such

as dopamine [11]. There are different types of alpha-synuclein, and the two types that have been associated with alpha-synuclein-induced toxicity are oligomers and fibrils [12].

Common to these proteins is that they begin their aggregation intracellularly.
When these protein aggregates inside neurons, it results in impaired brain function. These aggregates have a negative, toxic effect on the neurons in the brain [9, 10, 12].

1.1.1 Targeting alpha-synuclein

A loss of the dopaminergic neurons in PD is a disruption of several pathways caused by alpha-synuclein. The disturbed cellular pathways include mitochondrial function, oxidative stress, and endoplasmic reticulum (ER) stress, and disturbances in the autophagy and lysosomal pathway (see Figure 1). The mechanism of alpha-synuclein behind the toxic effect of alpha-synuclein is unknown, and current research focuses on stopping aggregation, among other things [12].

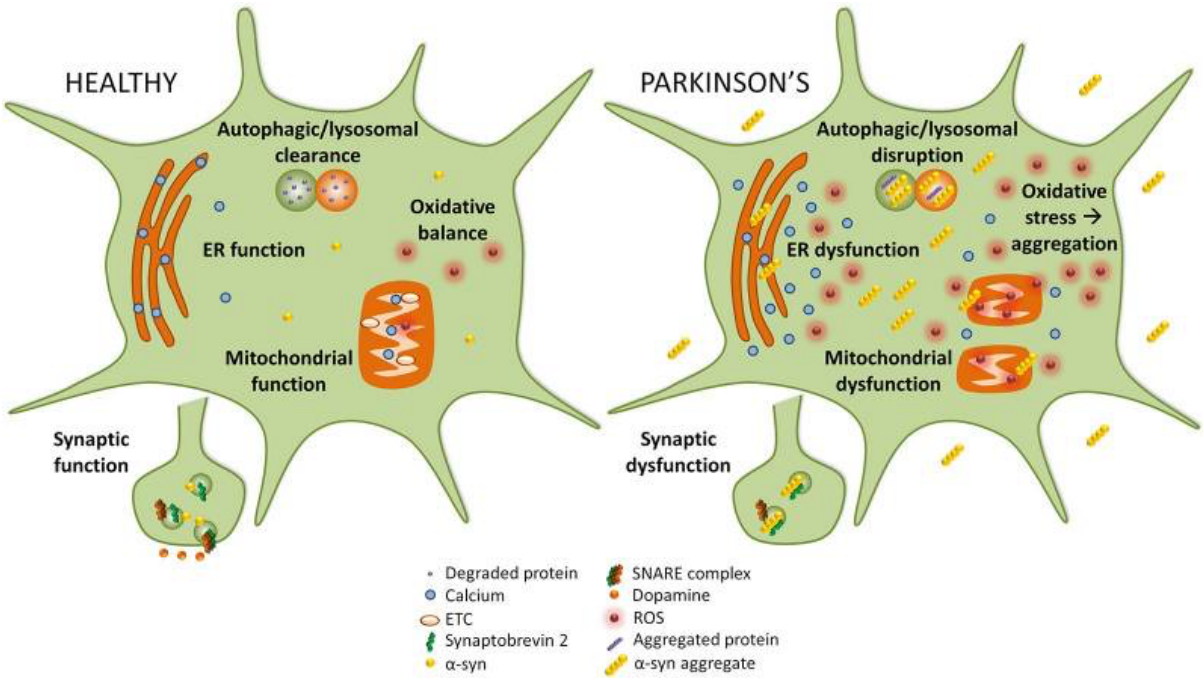

Figure 1: the image shows a healthy nerve cell and a PD affected nerve cell. In the nerve cell affected by PD, several cellular disorders lead to a decrease in the dopaminergic neurons [12].

1.2 Therapeutic strategies using protein based drugs

1.2.1 Antibody based therapeutics

Biological drugs, especially antibodies, have been shown to provide an excellent therapeutic strategy, as they have a specific binding to their target [13]. In addition, antibodies have a low probability of possible side effects. It makes the antibodies both safe and effective.

Antibodies are also large, which means that they can circulate in the body for a long time and, therefore, a long-term activity [13].

It has been shown that antibodies can recognize the toxic types of alpha-synuclein [14]. There is much research today on different strategies to reduce the formation of alpha-synuclein, and currently, there is no cure for the disease. Several strategies being researched today target alpha-synuclein by reducing its expression, inhibiting aggregation, and inhibiting the cellular uptake of alpha-synuclein [12].

The disadvantage is that the size of an antibody makes it challenging to penetrate cell membranes and other barriers in the body. It results in a low brain permeability for antibody-based therapeutics [13]. Thus, the challenge of using a protein-based biological drug targeting an intracellular target is that it becomes difficult to cross the cell membrane, due to its size. Treatments against intracellular targets are limited to small, selective molecules with a short half-life [13].

Furthermore, when developing and constructing antibody-based therapeutics, it becomes essential to know different parameters, as it can be costly in production if something goes wrong [13].

Today, no successful studies show that a protein based biological drug can enter the cell without being broken down before reaching its intracellular target [2]. Therefore, it becomes essential to develop a biological drug that can enter the neuron to bind intracellularly and stop the possible spread through a reduction [2, 3].

1.2.2 Single-chain fragment variable (scFvs)

Single-chain fragment variable (scFvs) has been shown to have an inhibitory effect against aggregation against the protein aggregates. A new generation of scFvs has been developed for use against, among others, alpha-synuclein [15].

scFvs are composed of the two variable regions of an antibody's heavy and light chains (VH and VL) [15]. The two domains are most linked by a flexible linker peptide composed of glycine and serine. Antibodies by contrast, have a Y-shaped structure consisting of two heavy chains and two light chains which are connected via disulfide bonds [16].

An advantage of scFvs, in contrast to other antibodies, is that it has a missing Fc portion. It indicates that there will be no phagocytosis, cell-mediated cytotoxicity, and no release of inflammatory mediators [17]. A disadvantage that generally applies to all antibodies is that they cannot pass through the cell membrane [18]. For these scFvs to exert their effect, they must be taken up to the brain and, through uptake mechanisms, reach their target [19].

1. 3 Uptake mechanism

1.3.1 Cell-penetrating peptide mediated uptake

Cell-penetrating peptides (CPPs) have promising potential because they can penetrate the cell membrane and translocate into the cytoplasm [20]. CPPs are classes of peptides that contain 5-30 amino acids. The function of the peptide is that it acts as a carrier. CPP's possible mechanisms are that it acts through direct penetration, endocytosis, and translocation [21, 22].

CPP has long been used for the delivery of several different peptides, proteins. Studies have also been performed on the delivery of siRNA with CPP to various cells. Several different cell-penetrating peptides have been identified, and they show a significant variation in length and polarity [20]. The cell-penetrating peptides are classified according to their chemical properties. They can either be cationic, amphipathic, and hydrophobic [21].

Drug delivery to the brain is usually low due to the blood brain barrier (BBB) which consists of dense endothelial cells, which is why there is a low permeability across the membrane. The mechanism behind CPP penetration through the cell is still unknown. However, there are three mechanisms for CPP to penetrate through the cell: direct penetration, translocation, and endocytosis. In addition, depending on the CPP family involved, the uptake mechanism may differ [21]. CPPs promotes penetration across the membrane, making it a promising option for future treatment for diseases caused in the central nervous system [22].

1.3.2 Adeno-associated virus (AAV) mediated uptake

Adeno-associated virus (AAV) is a viral vector, which has long been used against diseases of the central nervous system (CNS). There are several different serotypes for AAV, which also show tropism for neurons. The AAV-2 serotype has a high tropism for neurons [3].

Studies have used AAV to deliver proteins into the brain and these vectors have long been used to have a more direct effect against tau. The use of adeno-associated virus (AAV) against tau has shown a significant reduction in animal studies. A direct reduction of tau is obtained by AAV vectors encoding tau-directed antibodies, and hence they can deliver antibodies [23]. Studies that have used AVV have used intrabodies (iBs) that encode the tau-specific antibodies and scFv. AAV contains DNA encoding scFv, which is then delivered to the brain to reduce tau aggregation, [23, 24].

Several studies have used AAV containing DNA encoding for scFv, and the advantages are also due to the small size, and it can be easily wrapped in an AAV vector and provides a specific tissue penetration [24].

1.3.3 Dopamine transporter mediated uptake

The dopamine transporter (DAT) is located on the dopaminergic neuron, and its function is to be responsible for the reabsorption of dopamine in the synapse cleft. DAT has long been a target for the dopamine neurons responsible for several different diseases, including Parkinson's disease [25].

The DAT belongs to the SLC6 gene and has a high affinity for sodium/chloride channels, and is a transporter for, among other molecules, the neurotransmitter dopamine [25].

Drugs such as amphetamine have an exciting ability to enter the dopamine neuron through the internalization of DAT. The internalization of DAT can be achieved through regulating and stimulating DAT in the dopamine (DA) neuron [26]. Once inside the cell, it binds to trace amine-associated receptor 1 (TAAR1) and results in an increase in intracellular calcium. Increasing calcium levels lead to activation of GTPase and RhoA. It results in endocytosis of DAT present on the cell surface [26]. Internalization of DAT lead to the release of dopamine, but it says a lot about the fact that certain substances can enter the dopamine neuron [26].

1.3.4 Receptor- mediated uptake

Receptor-mediated transport (RMT) across the cell membrane has a high potential overcoming the BBB and a great chance to get more specific uptake [27]. On the cell surface, receptors are proteins that are capable of binding to specific molecules [28].

The mechanism behind RMT is that ligand binds to a transmembrane receptor located on the apical side of the membrane. It leads to endocytosis, by forming intracellular vesicles that contain the receptor-ligand complex that can eventually be taken to different destinations [27].

1.3.4.1 Transferrin receptor (TfR)

Transferrin receptor (TfR) has been used in studies of Alzheimer's disease and has been studied for a long time. It has been fused with scFv, which can further bind intracellularly. One study fused scFv with the C-terminal of TfR, designed as a cTfR-scFv. The complex is used to reduce amyloid-beta (Aβ) aggregate in the brain [27].

1.3.4.2 The low-density lipoprotein (LDL) receptor

The low-density lipoprotein (LDL) receptor (LDL-R) can deliver proteins from the blood to the central nervous system (CNS). The LDL-R belongs to a receptor family located on the cell surface. It binds primarily to lipoprotein complexes, which further leads to internalization and lysosomal degradation [29].

ESCRT stands for the endosomal sorting complex required for transport and involves several different processors. It can use membrane formation processes to transport the cell membrane to the lysosome [30].

The endosomal sorting complex (ESCRT) pathway has a facilitated function of transporting proteins from the endosome to the lysosome. A treatment study showed an endosomal degradation of the alpha-synuclein aggregates. Studies have shown that ESCRT can remove the alpha-synuclein by attaching sFv to the LDL-R-binding domain from apolipoprotein B (apo B) [17].

1.3.4.3 Fcγ-Receptor

Fcγ-Receptor is an IgG receptor and upon binding, the receptor triggers a cell activation, and an internalization of the complex occurs [31, 32].

Studies have shown that the antibodies can enter the cell through the internalization of the Fcy receptor. Once inside the cell, it has been shown to promote degradation of the tau aggregates by the lysosomal pathway [23].

1.3.4.4 Dopamine 1 receptor

The dopamine -1 receptor is a neuron-specific receptor found to a high degree in the brain and located on the cell surface of neurons [33].

It could be a potential candidate for specific uptake by the dopaminergic neuron.
The dopamine receptor belongs to the G-protein-coupled receptors and is found mainly in the central nervous system. These receptors are active in, among other processes, memory, learning, and cognition. There are several main groups of dopamine receptors. These can be divided into dopamine -1 receptors (D1), dopamine -2 receptors (D2) [33].

Dopamine 1 receptor endocytoses through caveolae. Caveolae are found in the plasma membrane and are a subset of lipid rafts. There have also been studies on whether palmitoylation may play a role in the internalization of the dopamine-1 receptor by a receptor-mediated pathway [34].

1.3.4.5 Acetylcholine receptor mediated uptake

The acetylcholine receptors are expressed on, among other cell types, neurons. There are several different types of acetylcholine receptors, including muscarinic receptors [35].

Those that have been shown to express the most in the brain are M1, M2, and M4. On the other hand, M3 and M5 are also expressed in the brain to a lesser extent. Acetylcholine receptors are involved in cholinergic functions such as memory and learning [36]

2. Aim

The purpose of the work is to find a strategy to reach the neurons intracellularly where the aggregation of alpha-synuclein occurs. The goal is to be able to design a biological drug that enters the neuron by investigating different uptake mechanisms.

3. Method

3.1 The database

To be able to answer the question, several different databases were used, including PubMed, Medline, Google scholar, to get a more and comprehensive subject overview.
Databases such as proteinatlas and UniProt were used to access biodistribution. The literature review was conducted between 18 January and 16 May 2021.

3.2 Search strategy

A systematic overview is performed. Google Scholar, which includes the database, will be used using a search strategy. A precaution has been taken when using Google scholar, only articles from secure sources have been included. The search strategy will be applied using relevant keywords, Mesh terms, in Google Scholar. The most relevant was chosen to adapt to the purpose of the project by using inclusion and exclusion criteria (see section 3.3)

Three different search strings were made to get an overview of the topic, and a summary was made with all three search hits made (See Figure 1).
As the subject is new, a manual search was also done via Google Scholar with the following search " antibodies and scFv and neurodegenerative diseases and neurons and neuronal uptake and intracellular target and intracellular". The search hit was 1390 articles, and the abstract were be read through.

Another search was done with following search "cell-penetrating peptide and neuronal uptake and intracellular target and neurodegenerative diseases and biological drug and antibodies and scfv" The search hit was 716 articles

To further investigates the dopamine transporter (DAT), the dopamine-1 receptors and acetylcholine receptor and endocytosis. A search was made to know more about how they work, how they are stimulated, and what can trigger endocytosis. The search hit was 128 articles.

Proteinatlas.org was used to access the biodistribution of LDL receptor, TfR receptor, dopamine-2 receptor, and acetylcholine receptor (M1). To get an overview of which tissues these receptors may be expressed in the selected section tissues.

3.3 Selection criteria

Studies included were complete research reports written in English. Articles published between the years 2000-2021 were included.

Because the purpose of the study is to reach the nerve cells intracellularly, studies with treatments outside the brain, studies whose purpose is not an intracellular goal were excluded. In addition, if the approach was not protein-based, they were also excluded because the purpose of the study is also to use protein-based drugs.

3.4 Data collection and analysis

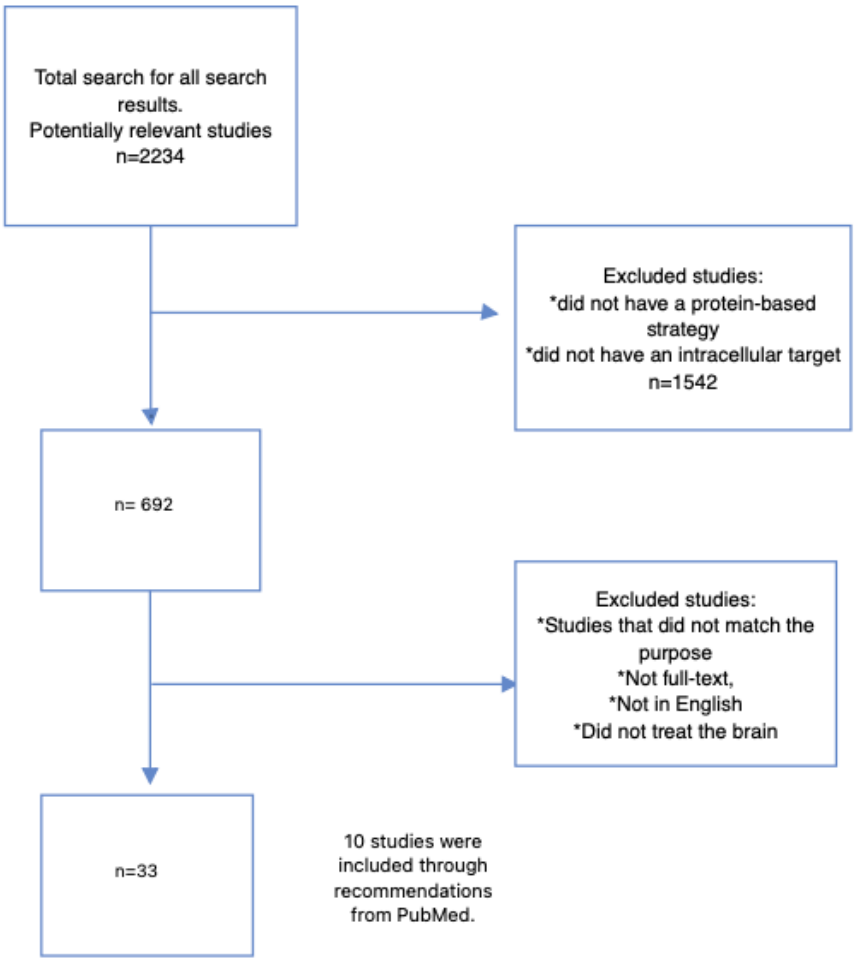

Figure 1: *flow chart for systematic overview*

4. Results

From the systematic overview, several published studies could be reviewed, and results could be obtained. All results will be presented in the form of a table and text form. The data show results from various receptors, cell-penetrating peptides, and adeno-associated viral vectors that were examined.

4.1 Neuron penetration and neuronal specificity.

Table 1: Published studies on different types of receptors, injected dose, and how much brain penetration and completed studies.

Type of receptor	Dose injected	Brain penetration	Intracellular target	Studies conducted	References
LDL receptor	No data	2-3% of injected dose	α-synuclein (α-syn)	Studies in mice were performed.	Spencer, et al. (2014)
FcγII/III	20 µg/ml.	No data	Tau	Studies in mice were performed.	Congdon et al, (2013)
TfR-receptor	1 mg/kg	>3% injected dose per gram	Amyloid-β (Aβ)	Chinese hamster ovary (CHO) cells	Boado, el al. (2010)
Fcγ-receptor	10 µg/ml	No data	Tau	In-vivo and in-vitro studies	Anderson et al, (2019)
LDL receptor	1 µM	No data	Lysosome	Cell studies were performed and animal studies in mice.	Scotti, et al. (2013)

Table 1 shows the results for the receptors examined. A summary of all studies for each receptor has been examined, where the data of injected dose was examined. Furthermore, how much was taken up in the brain is listed to know how effective it is.

In a neuron-specific uptake, it is essential to identify receptors expressed on neurons. In receptor-mediated uptake, several receptors can be examined, and it becomes a more specific uptake for a specific neuron. Among these may be the dopamine neuron.

Additionally, the neuron-specific receptors dopamine-1 and the acetylcholine receptors were examined. Results that could be examined were how the receptor could be internalized. A new study conducted by Underhill et al. showed that the stimulation of M1 and M5 has shown that DAT can be internalized to the dopamine neuron. When M1 / M2 is stimulated, phospholipase C can be released, leading to diacylglycerol (DAG) being released. Eventually, calcium will be released, leading to the activation of protein kinase C (PKC). Activation of PKC was shown to be necessary for DAT to be internalized [37].

Another study on the implementation of Kong et al. examined the dopamine-1 receptor. According to the study, dopamine-1 receptor is endocytosed by caveolae. Caveolae are found in the plasma membrane and are a subset of lipid rafts. Studies have also shown that palmitoylation may play a role in the internalization of the dopamine-1 receptor through a receptor-mediated pathway [34]. Palmitoylation is an acylation process, which can control several G protein-coupled receptors (GPCRs) functions. The process is initiated by the binding of palmitate, a long-chain fatty acid. Palmitate has a facilitating function with protein interactions [38].

Table 2: Published studies on different types of peptides/vector, injected dose, and how much brain penetration and completed studies.

Peptides/vector	Dose injected	Brain penetration	Intracellular target	Studies conducted	References
Penetratin	100 μL	No data	α-synuclein (α-syn)	Studies in mice were performed.	Spencer, et al. (2016)
Adeno-associated virus vector (AAV)	20–45 mg/kg	No data	Tau	Cell studies were performed and animal studies in mice.	Liu, et al. (2016)
Adeno-associated virus vector (AAV)	100 μm	No data	Tau	Studies in mice were performed.	Goodwin et al. (2021)
Adeno-associated	No data	No data	Huntingtin (htt)	Studies in mice were performed.	Butler et al. (2011)

virus vector (AAV 2)					
Tat 47–57	4.73 µl	No Data	No Data	Cell studies were performed and animal studies in mice.	Stalmans et al. (2015)
TP10-2	0.36 µl	No data	No data	Cell studies were performed and animal studies in mice.	Stalmans et al. (2015)
transportan 10 (TP10)	0.05 µl	No data	No Data	Cell studies were performed and animal studies in mice.	Stalmans et al. (2015)

Table 2 shows the results from peptides and vectors. A summary of all studies for both peptides and vectors was examined. The data was based on the type of peptide/vector used, how much was injected, and how much of the brain's injected dose was taken up. No data means that the published studies had no data on how much could be absorbed in the brain.

4.2 Neuronal specificity and safety assessment

A study was done based on the receptors' biodistribution to take a closer look at how neuron-specific they are, and an assessment can be made from a safety perspective.

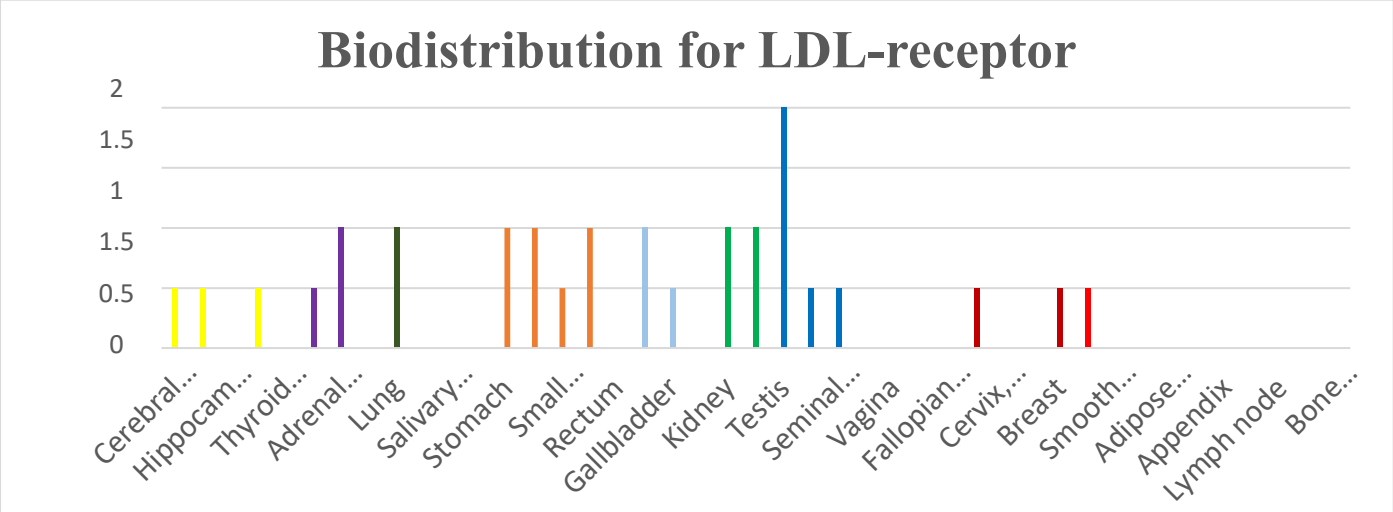

Figure 3: *Biodistribution for LDL receptor 2= high expression, 1= average level, 0.5= low level, 0= Not detected. Data available from proteinatlas.org*

Figure 3 shows the biodistribution of the Low-Density Lipoprotein (LLD) receptor.

The yellow color represents the biodistribution in the brain. The results show that the LDL receptor is also expressed in several organs other than the brain. A study on receptor biodistribution is essential from a safety perspective.

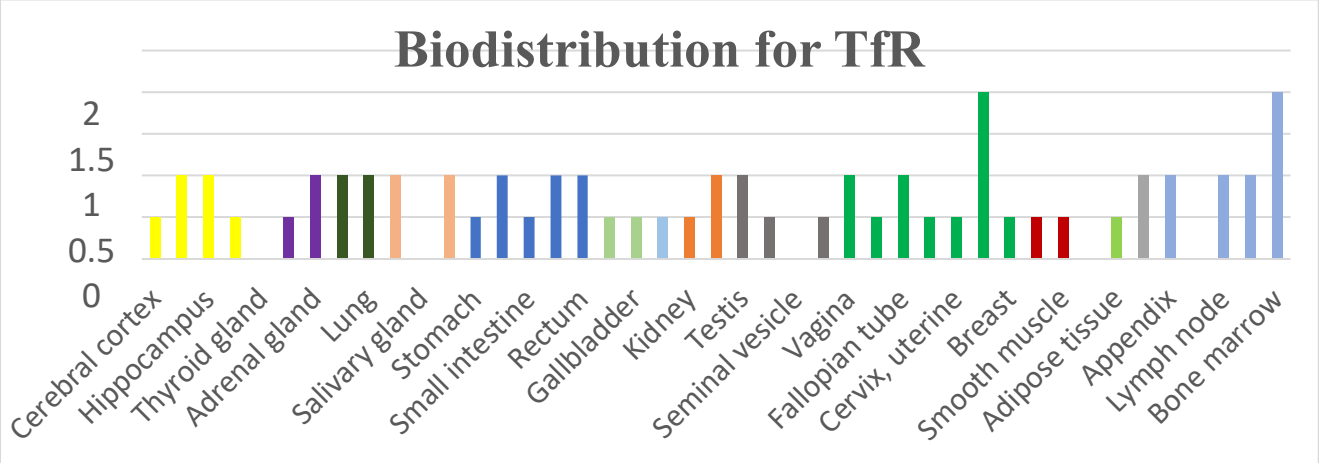

Figure 4: *biodistribution for TfR-receptor. 2= high expression, 1= average level, 0.5= low level, 0= Not detected. Data available from proteinatlas.org*

Figure 4 shows the biodistribution for transferrin receptor. Results for transferrin receptors also show that it is not only expressed in the brain, but in several organs.

Table 3: shows the biodistribution for several different types of Fcy receptor and their isoforms

Fcγ Receptors	**Isoform**	**Biodistribution**
FcγRI		Macrophages/ Monocytes / Glial cells / Neutrophil / Dendritic cell/ Neurons
FcγRII		
	FcγRIIA	Macrophages/ Monocytes / Dendritic cell/ Neutrophill/ Neurons
	FcγRIIB	Macrophages/ Monocytes / Dendritic cell/ Purkinje cells / Parvalbumin neurons
	FcγRIIC	Natural killer cell/ Neurons
FcγRIII		
	FcγRIII A	Macrophages/ Monocytes / Natural killer cell/ Neurons
	FcγRIII B	Neutrophil/ neurons

Table 3 shows that Fcy receptors are expressed on both glial cells and neurons. Fcy I is expressed on sensory neurons. However, Fcy II and Fcy III are not expressed on sensory neurons. Fcy IIb is expressed on Purkinje cells and parvalbumin neurons. The Fcy receptors

are also expressed on macrophages, monocytes, glial cells, neutrophils. Depending on the isoform, they can be expressed elsewhere in the body [39, 40]

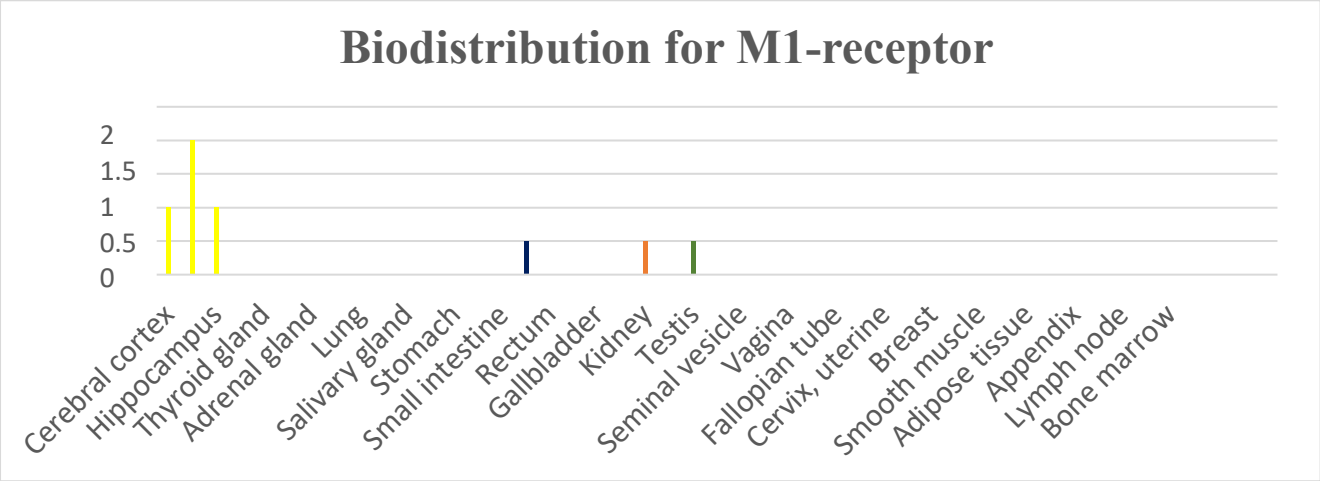

Figure 5: *Biodistribution for acetylcholine receptor, muscarine receptor M1. 2= high expression, 1= average level, 0.5= low level, 0= Not detected. Data available from proteinatlas.org*

Figure 5 shows an overview of where muscarinic acetylcholine receptor M1 is expressed. The muscarinic receptors 1 show a high expression in the brain and a low level in the kidneys, testis, and reticulum.

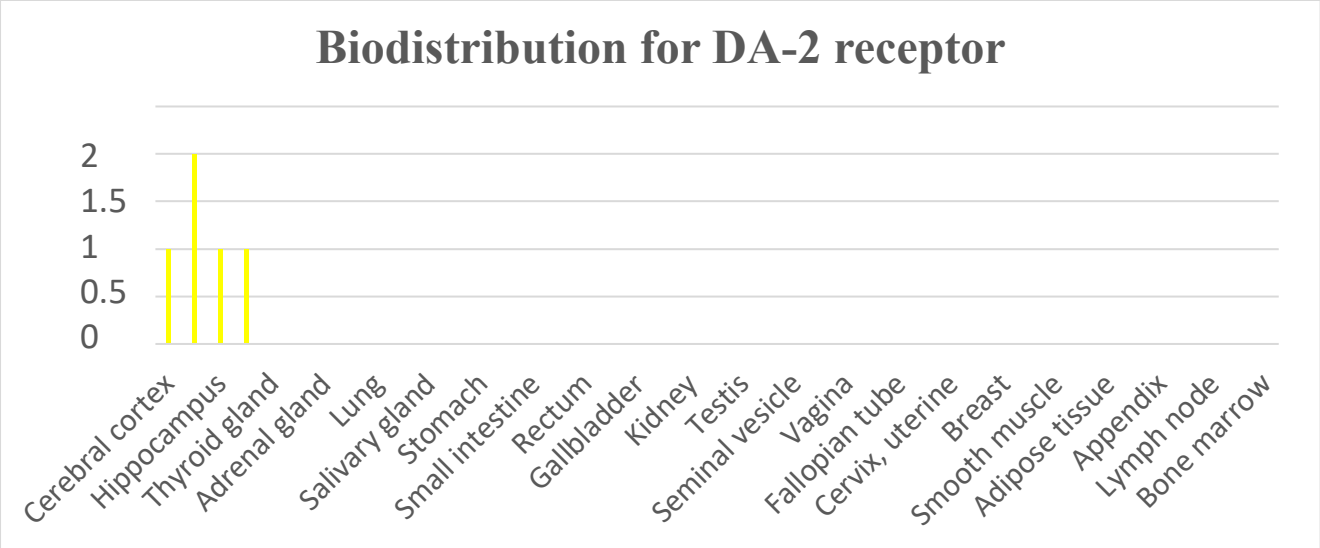

Figure 6: *Biodistribution for dopamine-2 receptor. 2= high expression, 1= average level, 0.5= low level, 0= Not detected. Data available from proteinatlas.org*

The biodistribution for D2 receptors shows a high expression in the brain, other places in the body have not been detected (Fig. 6). No data were available for the dopamine-1 receptor. However, D1 also shows neuron-specific expression.

5. Discussion

The purpose of the work is to find a strategy for reaching the nerve cells intracellularly where the aggregation of several different proteins takes place. By examining several different uptake mechanisms, e.g., receptors, peptides, and AAV, one can compare their penetration into the neuron and the neural specificity.

5.1 Neuron penetration

5.1.1 Low-density lipoprotein (LDL) receptor

Spencer et al. showed a modified form of scFv was made by fusing the LDL receptor-binding domain from apolipoprotein B (apo B). The low-density lipoprotein (LDL) receptor-binding domain was used to improve the penetration of scFv into the brain region. Table 1 shows that about 2-3% of the injecting dose reached the brain. However, no data was shown on how much was injected [17].

The receptor-ligand complex was transported into neurons through a so-called endosomal sorting complex required for transport (ESCRT) pathway. It results in lysosomal degradation of alpha-synuclein. The study showed that scFv had a reducing effect by targeting the oligomeric alpha-synuclein. Binding to LDL receptors facilitates penetrations into the CNS, cellular endocytosis and import into neuron [17].

Binding to lipoproteins showed endocytosis, resulting in autophagy to be degraded via the ESCRT pathway. When it comes to the ECRT pathway, it has a function that facilitates the transport of proteins from the endosome to the lysosome. The transport from the endosome to the lysosome is done using multivesicular bodies (MVBs) [17, 41].

In another study conducted by Scotti et al., There are two proteins PCSK9 and IDOL, that stimulate the LDL receptor to internalize by forming clathrin-coated pits.
When internalized, it is recognized by the ESCRT machinery, and the complex goes from the early endosome to the MVB to be further broken down in the lysosome [41].

According to Table 1, it can only reach the lysosome. The degradation takes place only in the lysosome and does not reach the target intracellularly. It means that it can bind to alpha-synuclein extracellularly before proceeding to the lysosome for degradation, but it is not suitable for targeting intracellular alpha-synuclein.

5.1.2 Transferrin receptor

In a study conducted by Boado R et al. TfRMAb was used to deliver scFv through the BBB to the brain. It was done by fusing the TfR with scFv and the complex is referred to as cTfRMAb [42]. Table 1 shows, > 3% of the injecting dose reached the brain. However, the target molecule is amyloid-beta, and these are found extracellularly [42].

The study showed only an extracellular binding, and the complex could not be taken up into the cell. Further studies are required to be able to use the transferrin receptor for the intracellular targets.

5.1.3 Fcy-Receptor

The uptake of the antibodies that the study examines took place through Fcy II / III receptors, and it is an energy-dependent process [40, 43].

Fcy receptors are a controversial type of receptor, with some studies suggesting that they do not have a high penetration into the brain [23]. Hence a poor alternative, and it would have been preferable to use an AAV [23]. In contrast, other studies show that they are expressed in neurons (see table 3).

It is worth mentioning that there are several types of Fcy receptors, and at the same time, they are divided into different isoforms (see table 3). Each isoform is expressed in certain types of neurons. What one could suggest is to study each receptor and isoform. Then, as previously mentioned, these can be expressed in other places in the body (see section 5.2.3)

Further modifications are required for the antibody to have a better affinity for the receptor. Modifications in the fc region of the antibody can lead to an increased affinity for neonatal FcRs [40].

5.1.2 Adeno-associated virus vector (AAV)

A study was conducted by Buttler et al. by performing an intrastriatal delivery of scFv to the N-terminal of htt. It was done using an adeno-associated virus vector (AAV2), and it has shown a reduction in the protein htt in the transgenic mice. However, it showed a short half-life [44].

In another recent study conducted by Godwin M et al., recombinant AVV2 was used to deliver scFv to its target. In neonatal mice, AAV was injected intraspinally, resulting in expression in the brainstem. [23]. There was no data on how much reaches the brain (see Table 2), even though it reached its intracellular target. The delivery of the antibodies takes place through cellular transduction.

AAV begins by interacting with a receptor on the cell surface, resulting in uptake into the cell through the endosome [45]. The problem with AAV is that the mechanism for the uptake of neurons is not complete. Another problem is that AAV has different serotypes, where each serotype has a unique tropism, which plays a significant role for future studies [46].

The challenge with AAV is that there is a lack of specific information about which proteins on the virus vector bind to which receptors on the cells.

5.2.1 Cell-penetrating peptide

For the cell-penetrating peptides, there is no exact uptake mechanism, and it may depend on the type of CPP, as they may be cationic, amphipathic, or hydrophobic.

Studies have been performed in which scFv was fused to Penetratin, resulting in a reduction in the CNS accumulating alpha-synuclein. It showed a greater significant uptake of the antibody into the brain. [47]. Penetratin can translocate cellular and nuclear lipid bilayers. The peptide can do it through a non-receptor medium, non-endocytic, and energy-independent manner.

Unlike the previous study conducted with the LDL-scFv through the ESCRT pathway, Penetratin can transport scFv both in and out of the neuron without any traditional pathway

[47]. As in the previous study with LDL receptor, an accumulation could be seen in the hippocampus and neocortex. It showed that to achieve a higher concentration of antibodies in the cytosol, specific binding of a receptor is required [14].

5.2 Neuronal specificity and safety assessment

5.2.1 Cell-penetrating peptide

Regarding Penetratin, no study was performed on what accumulation looked like on the rest of the body. Penetratin is a cell-penetrating peptide, and they are known to be absorbed throughout the body. Further studies will be needed to say with certainty that Penetratin also does not have a tissue-specific property.

5.2.2 Adeno-associated virus (AAV).

The studies included, no data was shown of the biodistribution for AAV. Studies indicate that the biodistribution for AVV can be in several organs in the body, such as the lungs, brain, liver, muscles, and heart. It does not make AAV tissue-specific and can be used for several different compounds of peptides to different tissues [48].

However, as mentioned earlier, there are different serotypes for AAV. The AAV2 serotype has been shown to have a tropism for neurons [3].

5.2.3 Receptors

Fcy receptors are expressed on, among other cell types, glia cells and primary neurons. More specifically, Fcy I receptors are found on sensory neurons, and Fcy IIb is found on Purkinje cells and parvalbumin neurons (see Table 3).

Modifications on antibodies can also lead to an increase in the affinity for FcRIIa / FcRIIb, and macrophages can be activated (see table 3). Activation of macrophages may lead to a risk of antibody-dependent cellular cytotoxicity (ADCC). More studies are needed to make Fcy safer and reduce any side effects. However, modifications in the antibody can lead to an improved affinity for the receptor, and that further studies on each isoform are much needed [40].

The biodistribution data shows that the receptors are expressed in the brain and found in other tissues. Both LDL receptor and Transferrin receptor are expressed in several different organs

(see figure 3 and 4). From a safety perspective, it is worth mentioning, as there is a possibility that it may cause other effects outside the brain.

The disadvantage of the receptors being expressed in several places in the body is that a smaller proportion may reach the brain, and higher concentrations reach places that are not desirable. In addition, there are no studies that examine what possible side effects it can give when receptors that are both neuron and tissue-specific are used.

The biodistribution of the dopamine-1 receptor shows that it is highly expressed in the brain. It makes it more tissue-specific and a good target for future studies.

Regarding the neuron-specific receptors dopamine-1 and acetylcholine receptors (M1), avoid intracellular signaling for the D1, M1 receptors and DAT.
Tau has been shown to increase calcium concentrations intracellularly [47], and so does the dopamine receptor when stimulated. When activated, it naturally stimulates calcium concentration. It can thus lead to an unwanted effect, possibly a side effect.
The dopamine receptor must be stimulated and endocytosed without signaling.

6. Limitations of study

The limitations of the work were that all published data were performed using different methods and had different units, making it difficult to compare data and study efficiency. Some types of studies used percentages in uptake. Others showed no data on how much entered the brain after injected dose. However, it was mentioned that there had been a reduction (%) of aggregated protein in the brain after drug injection.

The studies that have only performed cell studies make it difficult to say how credible the study is concerning whether one would give a systemic drug.

Some studies were not clear with which type(s) of receptors are used and their isoform. Hence it makes it challenging to make an individual assessment of each type of receptor.

7. Further studies

The biggest obstacle to further studies is that the pathogenesis of neurodegenerative diseases is unclear today. However, we know that there is an aggregation intracellularly, which worsens the course of the disease.

For future research or further studies, it is essential to note that the receptor/peptide should be neuron-specific, i.e., not being taken up elsewhere in the body. There may be a likelihood of unwanted effects. Thus, a more extensive study is needed on which neurons the receptors are specifically expressed. Receptors that can be expressed everywhere in a body become a safety issue, as there is a probability that it can lead to unwanted effects. Data that appear for all published studies say there is a reduction in the aggregates. It does not mention how much has been injected and the brain's proportion. Knowing the proportion of dose is essential as too much-injected dose can lead to some side effects on the experimental animals.

To have a better therapeutic strategy, it is necessary to know the exact uptake mechanism. For all published articles, it is necessary to know how the biological drug can further reach its intracellular target. Once the biological drug has been able to cross the cell membrane through a receptor-mediated uptake, it is essential to know how the antibody escapes from the endosome and know its causes. It is also essential to know which part of the biological drug prevents it from continuing to the cytosol but is broken down in the lysosome.

Most studies have been performed on cells, but also animal studies. It is a new concept, and no human studies have been conducted to date. It is essential to know that specific receptors expressed on animals are not expressed to the same extent in humans. Therefore, it becomes essential to know how far in the research they have come, what studies have been carried out.

Although the neuron-specific receptors, such as dopamine and acetylcholine, have not been tested, it may be a potential future solution if one could modify an antibody to bind to the dopamine-1 receptor, or the muscarinic acetylcholine receptors, without these eliciting any intracellular signaling.

8. Conclusion

In conclusion, there are not yet successful studies and further research is necessary. Treatments whose purpose has not been achieved and those expressed throughout the body,

there is a risk of side effects. Therefore, further studies on receptors, peptides, and vectors are required for the benefit to outweigh the risk. It may be relevant to consider alternative treatment, for example, the AVV and the neuron-specific receptors with further modifications, as these provide a tissue-specific uptake.

9. Ethical and sustainability aspects

The work was based on a systematic overview, which means no laboratory work took place, and no animal studies. However, there are ethical aspects to consider for future animal studies and an assessment of the benefits and risks. Animal studies can be used for future studies, where it is essential to consider the 3R: animal-free experimental models, reduce the number of animal experiments so that they do not suffer unnecessarily, and use gentle methods on the experimental animals.

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