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PRIMERJAVA PRIVZEMA HISTAMINA IN KVERCETINA V ASTROCITE NOVOROJENE PODGANE

DIFFERENCES IN HISTAMINE AND QUERCETIN UPTAKE IN NEONATAL RAT ASTROCYTES

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This Master's Thesis was performed at the Faculty of Medicine, Ljubljana, with the collaboration of Faculty of Pharmacy, Ljubljana, Slovenia. I worked under the mentorship of Professor Dr. Mojca Kržan and co-mentorship of Professor Dr. Sabina Passamonti.

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STATEMENT

I hereby declare that I have performed and written this Master's Thesis solely by myself under the mentorship of Professor Dr. Mojca Kržan, and co-mentorship of Professor Dr. Sabina Passamonti.

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Table of contents

ABSTRACT

The blood-brain barrier is a highly specialized barrier made of endothelial cells, pericytes and astrocytes. The barrier is only permeable for small molecules such as water, some gases and lipophilic molecules. The lipophilic molecules have to have some specific physico-chemical properties to be able to diffuse through the barrier; these properties are the following: MW < 500 Da, $\log P_{oct}$ from 2-4, and H-bond donors < 5. The results from some other studies are also showing that some lipophilic substances with the molecular weight as much as 5000 Da can cross the BBB and overpass the p-glycoprotein efflux system. But in general, lipophylic molecules that weight over 500 Da and water-soluble molecules cannot pass through the barrier, so they are transported across the barrier by a specialized carrier system. Transporters participate in influx and efflux of nutrients and xenobiotics; due to this fact, we can divide transporters into two big super families, SLC and ABC transporters. We were focused on SLC transporters, which participate in influx, while the ABC transporters mostly participate in efflux.
We compared two molecules, histamine and quercetin, and, more precisely, their uptake into neonatal rat astrocytes. They are both small lipophylic molecules, but since they are charged, we speculated that they both use the transport system to pass the barrier.

We did a study of uptake of the ^{3}H-Quercetin and compared it with results achieved in studies of histamine. First, we wanted to compare the kinetic characteristics of both ^{3}H-Quercetin and histamine, so we examined the concentration-dependence. We also wanted to examine the effects of bilirubin and some other substances on the ^{3}H-Quercetin uptake. Results show there are some differences in kinetic characteristics of both of them: while the process of histamine uptake is saturated, quercetin uptake does not reach saturation at concentrations used. Analysis of quercetin kinetic properties revealed two uptake sites, one with K_{m1} of 176 nM and V_{max} of 6 pmol/mg protein/min, and the other one with K_{m2} of 30.3 μM and V_{max2} of 60 pmol/min. The results of inhibition of quercetin uptake by bilirubin also show the possibility of blocking of two quercetin uptake sites. Thus, we confirmed that quercetin uses the transport system, but we would need to proceed with further researches to confirm the transporters involved.

RAZŠIRJENI POVZETEK

Krvno možganska pregrada ali hematoencefalna bariera je visoko specializirana pregrada med krvjo in možgani. Sestavljena je večinoma iz endotelijskih celic, vendar svoj delež doprinesejo tudi periciti in astrociti. Predvsem so nas zanimali slednji, ki predstavljajo 50 % vseh celic centralnega živčnega sistema in se nahajajo med nevroni in možganskimi kapilarami. Zadnje študije so pokazale, da astrociti sodelujejo v proizvodnji in pri sproščanju prostaglandinov, NO in arahidonske kisline. Nekatere študije so pokazale povezavo med astrociti in nekateri nevrološkimi boleznimi, kot so epilepsija, migrena, kap, vnetne bolezni, Alzheimerjeva bolezen, Parkinsova bolezen, amiotrofični lateralni sindrom in multipla skleroza. Po eni strani astrociti prizadenejo telo z večanjem prostih radikalov, kot so NO, peroskinitrit in superoksid, na drugi strani pa astrociti sodelujejo pri proizvodnji antioksidantov kot so superoksid dismutaza, askorbat in glutation.

Krvno-možganska pregrada je prepustna samo za manjše molekule, nekatere pline in lipofilne molekule določenih lastnosti. Lastnosti, ki jih mora imeti lipofilna molekula, da lahko prehaja skozi pregrado so: MM < 500 Da, $\log P_{okt}$ od 2-4, in donorskih H-vezi < 5. Torej najbolj primerne za prehod naj bi bile molekule z velikostjo od 400-600 Daltonov, vendar obstajajo študije, ki prikazujejo prehod skozi pregrado za substance tudi do 5000 Daltonov. Te študije sicer niso upoštevale sistema odtoka (efluks). Torej pregrado prehajajo večinoma lipofilne molekule, medtem ko v vodi topne molekule in nabite molekule, ter večje lipofilne molekule ne prehajajo prosto difuzno v možgane, temveč uporabljajo posebne transportne sisteme.

Transportni sistem se na grobo deli na dve veliki družini in sicer na SLC ter ABC transporterje, pri čemer slednji sodelujejo večinoma pri odtoku snovi, zato smo se osredotočili le na SLC transporterje. SLC družino sestavlja 40 različnih proteinov, ki prenašajo različne snovi, od aminokislin pa do vitaminov. Znanih je osem članov SLC transporterjev: OATP, OCT, OCTN, OAT, PEPT, CNT, ENT in MATE. OATP in OAT sta transporterja , ki sta odgovorna za transport anionskih molekul, OCT in OCTN za kationske molekule, PEPT za dipeptide in tripeptide, CNT in ENT za nukleozide ter MATE za ksenobiotike.

Pri naši diplomski nalogi smo se ukvarjali s primerjavo dveh molekul in sicer histamina ter kvercetina.

Histamin je pomemben biogeni amin v procesu spanja-budnosti, spomina, apetita, čustev, učenja in tudi določenih bolezni kot so Alzheimerjeva bolezen, depresija, epilepsija in shizofrenija. Gre za dvojno protonirano molekulo, kar pomeni da ne more prosto prehajati krvno-možganske pregrade, temveč potrebuje transporter. Po zadnjih študijah naj bi bil za transport najprimernejši protein OCT. Znani so trije člani OCT transporterjev, pri čemer je izražanje OCT1 in OCT2 v možganih omejeno, zato predvidevajo, da naj bi bil za transport odgovoren OCT3.

Druga molekula, s katero smo se ukvarjali je kvercetin, zelo pomemben antioksidant. Spada med skupino flavonoidov in sicer med flavonole. Pomemben je zaradi številnih ugodnih lastnosti na organizem, predvsem zaradi svojih protivnetnih, antikarcinogenih in antioksidativnih učinkov. Gre za lipofilno molekulo, ki ima velikost 302 Daltonov, vendar kljub temu ne prehaja krvno-možganske pregrade. Razlog je v tem, da je tako kot histamin tudi kvercetin protonirana molekula. Zadnje študije kažejo, da obstaja možnost, da kvercetin prehaja v možgane s pomočjo transmembranskega proteina bilitranslokaze.

Pri diplomski nalogi smo želeli dokazati, da se kvercetin prenaša skozi krvno-možgansko pregrado s pomočjo transportnega sistema tako kot histamin in da so kinetične lastnosti privzema histamina in kvercetina različne.

Pri kinetičnih lastnostih smo preučevali ali je privzem kvercetina odvisen od temperature in koncentracije, nato pa rezultate primerjali s podatki za histamin, ki so bili pridobljeni iz drugih študij. Dokazali smo, da so kinetične lastnosti privzema kvercetina in histamina različne. Oba privzema sta časovno odvisna, vendar je privzem kvercetina bistveno hitrejši od privzema histamina. Ugotovili smo tudi, da sta oba procesa koncentracijsko odvisna, pri čemer je privzem histamina nasitljiv proces, medtem ko pri privzemu kvercetina tega ni bilo opaziti. Preverjali smo privzem pri dveh različnih temperaturah: 4°C in 37°C. Pri 4°C ne poteka ATP-odvisni proces, le preprosta ali olajšana difuzija. Rezultati so pokazali, da pri kvercetinu za razliko od histamina ne gre za ATP-odvisni proces.

V drugem delu diplomske naloge smo se ukvarjali z vplivom določenih snovi na privzem kvercetina. Kvercetin ima podobne fizikalno-kemijske lastnosti kot bilirubin, zato nas je zanimal vpliv različnih koncentracij bilirubina na sam privzem kvercetina pri čemer smo

ugotovili, da pri zelo nizkih koncentracijah bilirubin zavira privzem kvercetina. Pri koncentraciji 70nm nismo opazili vpliva, medtem ko višje koncentracije zopet zavirajo privzem kvercetina. To smo povezali z morebitno vezavo kvercetina na bilitranslokazo, ki je sicer transmembranski prenašalni protein, specifičen za bilirubin. Rezultati so nam pokazali, da se kvercetin prenaša skozi membrano astrocitov s pomočjo dveh prenašalnih molekul.

Analiza privzema kvercetina je pokazala, da obstajata dve vezavni mesti: prvo s K_{m1} 176 nM in V_{max} 6 pmol/mg protein/min in drugo s K_{m2} 30.3 μM in V_{max2} 60 pmol/min. Prav tako so rezultati zaviranja privzema kvercetina z bilirubinom pokazali možnost zaviranja obeh vezavnih mest.

Privzem kvercetina pomočjo prenašalcev smo preverili z vplivom natrijevih, kloridnih in kalijevih ionov ter ouabaina.. Ugotovili smo, da pri zamenjavi natrijevih soli s holinom kloridom ni prišlo do spremembe privzema kvercetina, zatorej prenos kvercetina ni odvisen od natrija. Presežek K^+ poveča privzem kvercetina. Ugotovili smo, da je privzem kvercetina odvisen tudi od Cl^-. Iz tega lahko sklepamo, da privzem kvercetina ni odvisen od Na^+ in K^+ , ki sta značilna za ATP-odvisni proces.

Potrdili smo, da se kvercetin prenaša s pomočjo transportnega sistema, bodisi s pomočjo bilitranslokaze bodisi s pomočjo enega izmed OAT prenašalcev. Natančnejše ugotavljanje tega prenašalca, zahteva nadaljevanje teh raziskav.

ABBREVIATIONS

AF-6	Afadin-6
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle medium
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
P-gp	P-glycoprotein
Ra1A	Ras family small GTP binding protein
Raf-1	Rapidly Accelerated Fibrosarcoma - 1
RhoA	Ras homolog gene family
TGF-α	Transforming growth factor alpha
ZO-1	zona occludens-1
ZO-2	zona occludens-2
ZO-3	zona occludens-3
ZONAB	zonula occludens 1 (ZO-1)–associated nucleic acid binding protein

1.INTRODUCTION

1.1. BLOOD-BRAIN BARRIER

In late 19th century, Paul Erlich and his students suggested the existence of a barrier separating the circulation from the brain tissue. Paul Erlich′s student could not stain the brain when injecting the aniline dye into the blood vessel, but succeeded after having injected aniline dye directly into the cerebrospinal fluid. In the later case, the rest of the body of the test animal was not stained. In spite of discovering the function of the blood-brain barrier (BBB), the researchers from Paul Erlich′s group could not confirm the morphological proof for the existence of a barrier between blood and brain. This was not possible until electron microscope techniques become available.

The results achieved by Dawson and his group in 1940 were more convincing; they found out that endothelial cells regulated the passage of proteins from blood to brain. The research of transport of different compounds into the brain continued in the 1970s by Oldendorf (1) who also proposed the name – blood brain barrier.

Nowadays, we know that the BBB is a highly specialized barrier made of endothelial cells, astrocytes and pericytes (Figure 1) (2) that controls the passage of solutes, peptides and proteins between the blood and the brain. In general, it is true that the BBB mostly consists of endothelial cells, but for the BBB to function normally, astrocytes and pericytes are necessary (3).

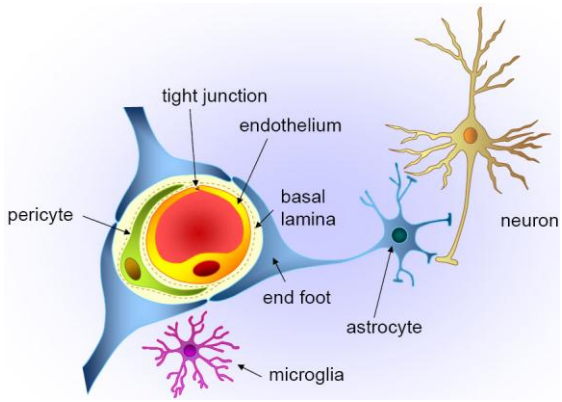

Figure 1: Structure of BBB (adapted from (4))

1.1.1. ENDOTHELIAL CELLS

Endothelial cells are thin and flat cells, interconnected by tight junctions that we can observe in Figure 2. These tight junctions are responsible for high endothelial electrical resistance and low paracellular permeability. Nevertheless, endothelial cells express a vast number of different membrane transporters, which regulate the influx and efflux of different substances (5).

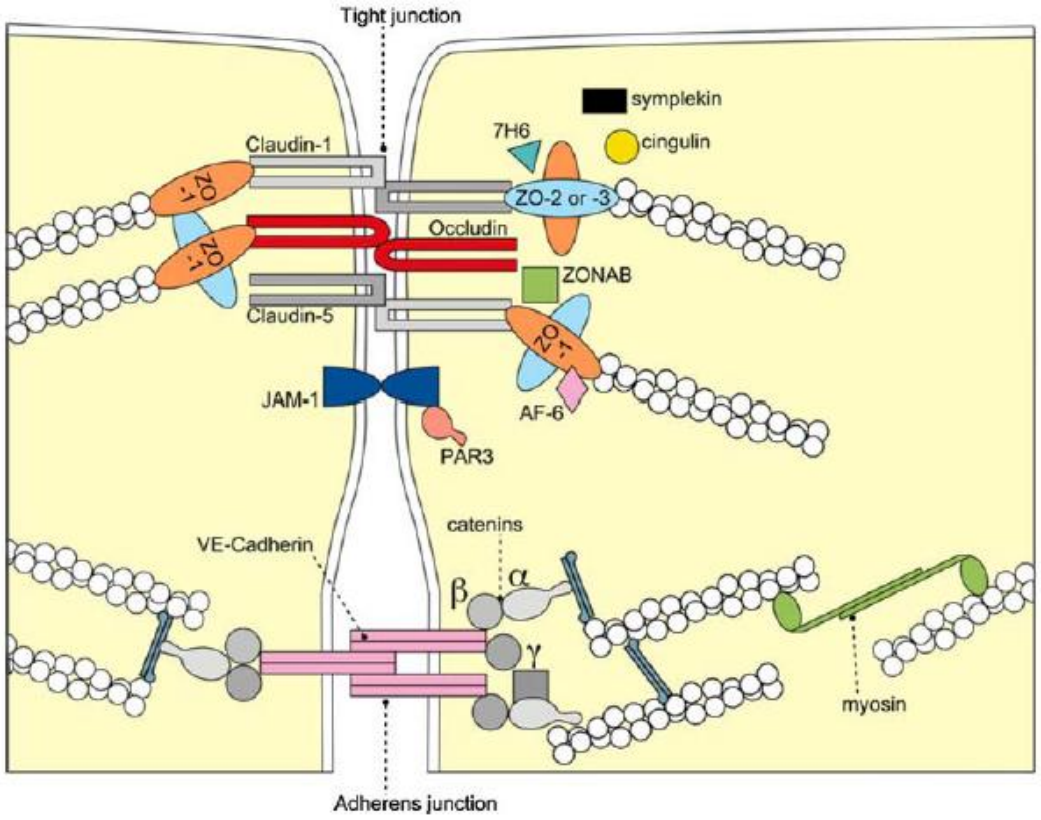

Figure 2: Tight junction. Adapted from (6)

The tight junction acts as a ″barrier″ responsible for the organisation of different functions: cell polarity, cell proliferation, morphogenesis and differentiation. The tight junction is made by a network of different proteins like ZO-1, ZO-2, ZO-3, AF-6 and cingulin (Figure 2). The proper function of BBB is regulated by proteins like ZONAB, RhoA, RalA and Raf-1, which are responsible for the regulation of barrier function and gene transcription (6). The important proteins of endothelial cell membrane are claudins, JAM and occludin (7).

- **Claudins**

  There are 24 subtypes of claudin transmembrane phosphoproteins. Each claudin has four transmembrane domains. Claudins, JAM and occludin form a tight junction and prevent the passage of molecules with molecular mass over 600 Da. The expression of claudin-1 is altered in various neurological diseases.

- **Occludin**

  Occludin is also a four transmembrane domain phosphoprotein, localized parallel to claudin. An interesting fact is that occludin is not presented in newborns; it appears later, when brain matures. Occludin has an important role in the regulation of the BBB permeability.

- **JAM (Junctional adhesion molecule)**

  These are glycoproteins and immunoglobulins that exist in three different types. They are also important for the regulation of the BBB permeability (7).

1.1.2. PERICYTES

Pericytes are present in the duplication of the basement membrane and in the proximity of endothelial cells, meaning that they can communicate with endothelial cells by means of a direct physical contact. Pericytes are able to synthesize many active substances and therefore play an important role in the following actions:

- regulation of blood flow and auto-regulation of blood vessels,
- angiogenesis of the brain,
- BBB differentiation,
- developing and maintaining the tight junctions,
- launching the extrinsic pathway of coagulation after brain injuries
- inflammatory processes and endothelial proliferation (8) (7).

1.1.3. ASTROCYTES

Astrocytes are specialized glial cells that represent 50% of all cells in CNS and are positioned between neurons and brain capillaries. They are the only cell types expressing GFAP (2), a sensitive and a reliable marker that is detected in almost all astrocytes.

Recent studies show that astrocytes participate in producing and releasing prostaglandins, NO and arachidonic acid, mediators that affect the blood vessel diameter and blood flow in CNS. Astrocytes are also responsible for releasing agents which support the formation of tight junctions, such as TGF-α and GDNF (9).

Function of astrocytes is different through life, as we see in the table 1 bellow.

Table 1: Function of astrocytes (adapted from (2))

Developmental phase	The guidance of growing neurons Trophic factor synthesis Synapse formation
Normal adulthood	Maintenance of homeostasis Influence on synaptic plasticity Tripartite synapse
After brain injury	Swelling of astrocytes Reactive gliosis

There are four known types of astrocytes:

- Protoplasmatic astrocyte: the most represented astrocyte in the CNS gray matter
- Interlaminar astrocyte: important in processes that create sets of GFAP fibres
- Polarized astrocyte: very similar to neurons, they can only be found in human brain
- Fibrous astrocyte: located in white matter, probably important for the support of glial cells (9) (10)

Certain studies show a correlation between astrocytes and some neurological diseases, such as epilepsy, migraine, stroke, inflammatory diseases, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis. They are involved in both of

the mechanisms, damage and neuroprotective (9) (11). On one hand, astrocytes damage the body with increased production and release of free radicals, such as NO, peroxynitrite and superoxide, but on the other hand, astrocytes also help with producing antioxidants, such as superoxide dismutases, ascorbate and glutathione, and taking up the excess of potassium ions and excitatory neurotransmitters like glutamate, thus preventing neurons from excitotoxic injuries (11).

1.2. BBB TRANSPORTERS

1.2.1. TRANSPORT THROUGH BBB

In 1999, Levin researched p-glycoprotein efflux system functions and found out that substances with molecular weight under 400-600 Dalton can cross the BBB by a simple diffusion. However, the results of other studies showed that some substances with molecular weight as much as 5000 Da can cross the BBB and overpass p-glycoprotein efflux system. It was also found that substances with a molecular mass over 500 Da cannot cross the BBB unless they are transported across the BBB by a specialized carrier system.

Transport systems are necessary for penetration of exogenous substances, such as essential amino acids (tyrosine, leucine, tryptophan), nucleic acids, glucose, free fatty acids (arachidonate, palmitate), minerals (Fe, Zn, Mg) and electrolytes (K, Na), substances essential for a normal functioning of the mammalian brain, (1).

About 100% of heavy molecules and about 98% of small molecules do not diffuse through the BBB. Histamine, for example, which is a molecule with a molecular mass of 100 Dalton, cannot diffuse through an intact BBB (12).

The BBB is a lipid-soluble barrier, permeable only for highly lipophilic molecules which pass the barrier by means of simple diffusion. All water-soluble molecules and electrically charged molecules cannot diffuse through BBB but have to use a carrier-operated system instead.

In general, there are four ways of transport through brain: ATP-driven active transport, facilitated diffusion, transcytosis and simple diffusion (13). The complexity of transport through the BBB is shown in Figure 3.

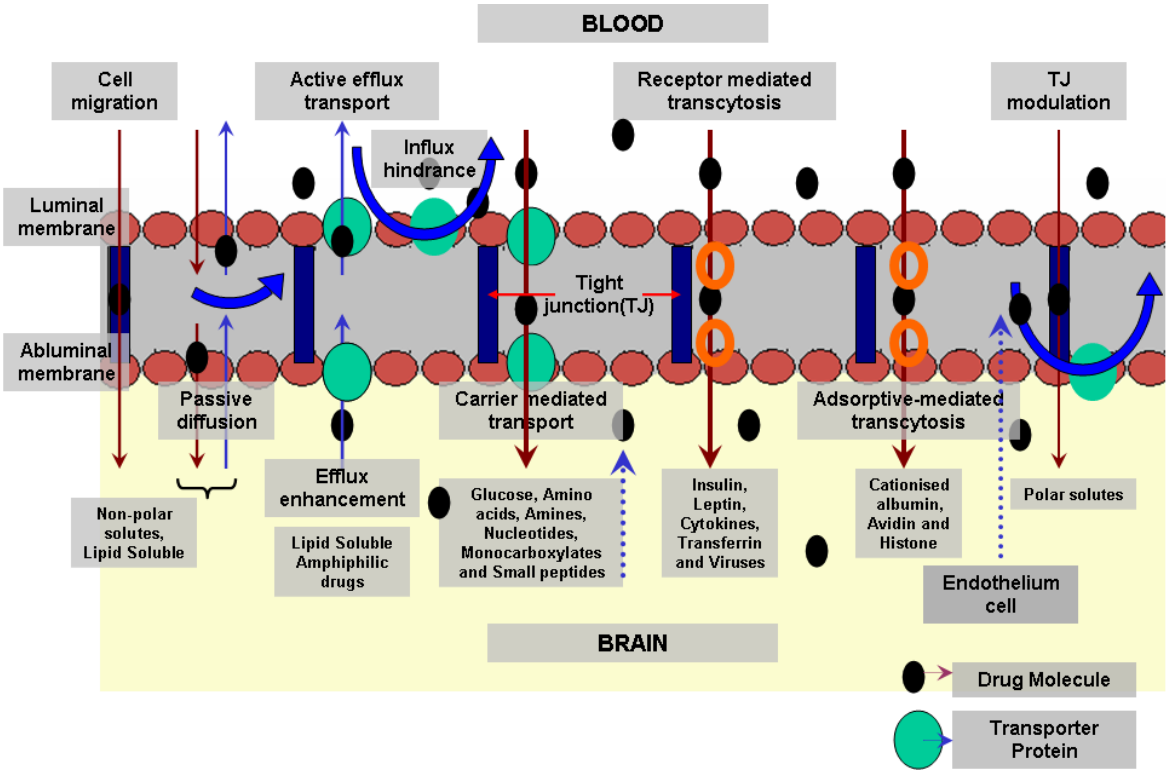

Figure 3: Transport through BBB. Adapted from (13)

Passive transport or simple diffusion in the BBB is a spontaneous process, where lipophilic molecules move across the membrane without the use of free or chemical energy. During the process, the entropy increases (14). Physico-chemical properties of molecules that are able to diffuse through the BBB are the following: MW < 500 Da, $\log P_{oct}$ from 2-4, and H-bond donors < 5 (15).

Active transport is an energy-consuming process and needs coupling of the hydrolysis of ATP as the energy source. ATP coupling can be primary or secondary. In primary coupling, transporters are carrying molecules against their chemical or electrical gradient and for this process, they hydrolyze ATP. In secondary coupling, transporters use ion gradients as the driving force and molecules are transported against an electrochemical gradient (14).

Transporter molecules participate in the influx and the efflux of nutrients and xenobiotics. Transporter proteins belong to two superfamilies of biomolecules: solute linked carrier (SLC) and ATP binding cassette transporters (ABC) (16). The main difference between these two transporter superfamilies is that the SLC transporters are important for the influx and the uptake of nutrients in the brain, while ABC transporters are important for the efflux of substrates. There is also a difference about the type of the transport used: SLC transporters are using secondary and tertiary transport, while ABC transporters are using primary transport. In our research, we will mainly focus on SLC transporters (17).

1.2.2. SLC TRANSPORTERS

SLC family includes about 40 different proteins that carry solutes, from amino-acids to ions and vitamins. SLC family includes exchangers which need no ATP for their function (18). The expression of SLC proteins is not limited to brain, but it is found also in placenta, liver, kidney, blood-testis barrier and small intestine (19).

There are known eight typical members of SLC family:

- Organic Anion Transporting Polypeptides (OATP)
- Organic Cation Transporters (OCT)
- Organic Cation/Carnitine Transporters (OCTN)
- Organic Anion Transporters (OAT)
- Peptide Transporters (PEPT)
- Concentrative Nucleoside Transporters (CNT)
- Equilibrative Nucleoside Transporters (ENT)
- Multidrug and Toxin Extrusion Transporters (MATE) (20)

For every member of SLC family are important some typical substrate, as we see in table 3.

Table 2: Substrates for SLC transporters (adapted from (20))

OATP1A2	Sulfobromophthalein, BQ-123, cholic acid, dehydroepiandrosterone sulfate, deltophorin II, DPDPE, estrone-3-sulfate, fexofenadine, glycocholate, levofloxacin, methotrexate, microcystin-LR, ouabain, prostaglandin E2, rosuvastatin, saquinavir, taurocholate, thyroxine, triiodothyronine
OATP1B1	Benzylpenicillin, bilirubin and its conjugates, bosentan, BQ-123, bromosulfophthalein, caspofungin,

	cerivastatin, cholic acid, dehydroepiandrosterone sulfate, DPDPE, estradiol 17_-glucuronide, estrone-3-sulfate, fluvastatin, glycocholate, irinotecan,(SN38 metabolite), leukotriene C4, microcystin-LR, olmesartan, phalloidin, pravastatin, prostaglandin E2, rifampicin,,rifampin, rosuvastatin, taurocholate, thromboxane B2, thyroxine, triiodothyronine, troglitazone sulfate, valsartan
OATP1B3	Bilirubin conjugates, bosentan, sulfobromophthalein, BQ-123, cholecystokinin-8, dehydroepiandrosterone sulfate, deltorphin II, digoxin, DPDPE, docetaxel, estradiol 17_-glucuronide, fexofenadine, fluvastatin, glycocholate, irinotecan (SN38 metabolite), leukotriene C4, microcystin-LR, olmesartan, ouabain, paclitaxel, phalloidin, rifampicin, rifampin, rosuvastatin, taurocholate, telmisartan, thyroxine, triiodothyronine, valsartan
OCT1	Acetylcholine, acyclovir, cimetidine, choline, dopamine, famotidine, ganciclovir, lamivudine, metformin, *N*-methylnicotinamide, 1-methyl-4-phenylpyridinium, quinine, ranitidine, serotonin, spermine, spermidine, tetraethylammonium, zalcitabine, OCT2 Acetylcholine, amantadine, cimetidine, cisplatin, choline, dopamine, epinephrine, histamine, lamivudine, memantine, metformin, 1-methyl-4-phenylpyridinium, *N*-methylnicotinamide, norepinephrine, paraquat, prostaglandin E2, prostaglandin, F2, quinine, ranitidine, serotonin, tetraethylammonium, zalcitabine
OCT3	Atropine, dopamine, epinephrine, etilefrine, guanidine, histamine, 1-methyl-4-phenylpyridinium, tetraethylammonium
OCTN1	L-Carnitine, ergothioneine, pyrilamine, quinidine, quinine, tetraethylammonium, verapamil
OCTN2	L-Carnitine, cephaloridine, mildronate, pyrilamine, quinidine, spironolactone, tetraethylammonium, valproic acid, verapamil
OAT1	Acetylsalicylate, acyclovir, adefovir, *p*-aminohippurate, cephaloridine, cidofovir, cimetidine, cyclic AMP and GMP, didanosine, edaravone sulfate, furosemide, ganciclovir, indoxyl sulfate, indomethacin, _-ketoglutarate, lamivudine, methotrexate, ochratoxin A, penicillin G, prostaglandins E2 and F2, salicylate, stavudine, tetracycline, trifluridine, urate, zidovudine, zalcitabine
OAT2	*p*-Aminohippurate, acetylsalicylate, allopurinol, bumetanide, cyclic AMP, dehydroepiandrosterone sulfate, estrone-3-sulfate, 5-fluorouracil, glutarate, _-ketoglutarate, methotrexate, paclitaxel, prostaglandins E2 and F2_, ochratoxin A, salicylate, tetracycline, valproic acid, zidovudine
OAT3	Allopurinol, *p*-aminohippurate, benzylpenicillin, L-carnitine, cefazolin, cephaloridine, cholic acid, cimetidine, cortisol, dehydroepiandrosterone sulfate, edaravone sulfate, estrone-3-sulfate, famotidine, 5-fluorouracil, glutarate, glutathione, glycocholate, indoxyl sulfate, methotrexate, 6-mercaptopurine, ochratoxin A, pravastatin, prostaglandins E2 and F2, rosuvastatin, taurocholate, tetracycline, urate, valacyclovir, zidovudine
OAT4	*p*-Aminohippurate, dehydroepiandrosterone sulfate, estrone-3-sulfate, glutarate, indoxyl sulfate, ochratoxin A, tetracycline,zidovudine
PEPT1	5-Aminolevulinic acid, bestatin, cefadroxil, ceftibuten, cefixime, cephradine, cephalexin, glycylsarcosine

PEPT2	5-Aminolevulinic acid, bestatin, cefadroxil, glycylsarcosine, kyotorphin
CNT1	Adenosine, cladribine, cytarabine, fialuridine, 5-fluorouridine, gemcitabine, stavudine, thymidine, uridine, zalcitabine, zidovudine
CNT2	Adenosine, cladribine, clofarabine, cytidine, didanosine, fialuridine, 5-fluorouridine, formycin B, inosine, guanosine, ribavirin, tiazofurin, uridine
CNT3	Adenosine, benzamide riboside, cladribine, clofarabine, cytarabine, cytidine, didanosine, fludarabine, 5-fluorouridine, gemcitabine, guanosine, inosine, 6-mercaptopurine, ribavirin, uridine, 6-thioguanine, tiazofurin, thymidine, zalcitabine, zebularine, zidovudine
ENT1	Adenosine, cladribine, clofarabine, cytidine, fialuridine, gemcitabine, guanosine, ribavirin, thymidine, tiazofurin, uridine
ENT2	Adenine, adenosine, clofarabine, cytidine, fialuridine, gemcitabine, guanine, guanosine, hypoxanthine, inosine, thymidine, tiazofurin, uridine
ENT3	Adenine, adenosine, cladribine, fludarabine, guanosine, inosine, thymidine, uridine, zebularine, zidovudine
MATE1	Acyclovir, cephalexin, cephradine, cimetidine, creatinine, estrone sulfate, ganciclovir, guanidine, 1-methyl-4-phenylpyridinium, metformin, oxaliplatin, paraquat, procainamide, tenofovir, tetraethylammonium, thiamine, topotecan
MATE2-K	Acyclovir, cimetidine, creatinine, estrone sulfate, ganciclovir, guanidine, metformin, 1-methyl-4-phenylpyridinium, 1-methylnicotinamide, oxaliplatin, procainamide, tetraethylammonium, thiamine, topotecan

Organic Anion Transporting Polypeptides (OATP)

As we see up in Table 2, OATPs are responsible for transport of anionic peptides, thyroid hormones, steroid conjugates and different xenobiotics. They are membrane proteins with 12 transmembrane helices. Up to now there are 11 known members of OATP transporters: OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1, OATP5A1 and OATP6A1, out of which only. OATP1A2, 1A4 and 1C1 are expressed in brain. OATP1C1 is highly expressed in human and murine brain, while the expression of other members is a species-dependent. It is known that OATP transporters are typical uptake transporters, with ability to transport the substrates bidirectionally. OATP1B1 and OATP1B3 are classical bidirectional transporters (20) (7).

Organic Cation Transporters (OCT)

The OCT family has three members: OCT1, OCT2 and OCT3. OCT1 is expressed mainly in liver, but it is also found in heart, brain, kidney, skeletal muscle and placenta as we can see in Figure 4. OCT2 is specific for kidney, but is also found in cerebral cortex, hippocampus and lungs. OCT3 is expressed in liver, placenta, skeletal muscle and kidney, and slightly in lung, brain and heart. OCT-mediated transport is not ATP- or Na^+-dependent (21).

Organic Cation/Carnitine Transporters (OCTN)

OCTNs are important in mediating the influx carnitine. Carnitine helps to transfer fatty acids from cytosol to mitochondria and where they are degraded by β-oxidation. In addition to carnitine, they can also transport other cations as we can see in Table 3. We know two members of OCTN: OCTN1 and OCTN2. The first one is uniporter and antiporter, so the transport can leads in both ways. The second one is just uniporter, so the transport leads just in one way (20).

Organic Anion Transporters (OAT)

There are 10 members of OATs. OAT1 is specific for kidney, OAT2 for liver and OAT3 for brain (7). Transport is able due to Na^+ and dicarboxylate gradient made by the sodium-dicarboxylate co-transporter and the Na^+-K^+ ATPase. This helps the transport of organic anions against membrane potential (20).

Peptide Transporters (PEPT)

PEPTs are divided into PEPT1 and PEPT2, where the PEPT1 is expressed in the intestine while PEPT 2 is expressed in the renal cortex. PEPT1 is important for transporting of dipeptides and tripeptides, but it does not carry free amino acids or peptides with more than 3 amino groups (22).

Concentrative Nucleoside Transporters (CNT)

CNTs are responsible for transport of endogenous nucleoside and nucleoside analog drugs. They are expressed in the intestine, BBB, kidney and liver. There are three members of CNT: CNT1, that transports pyrimidines, CNT2, which take care of transport of purines and CNT3, that transports both, purines and pyrimidines (22) (20).

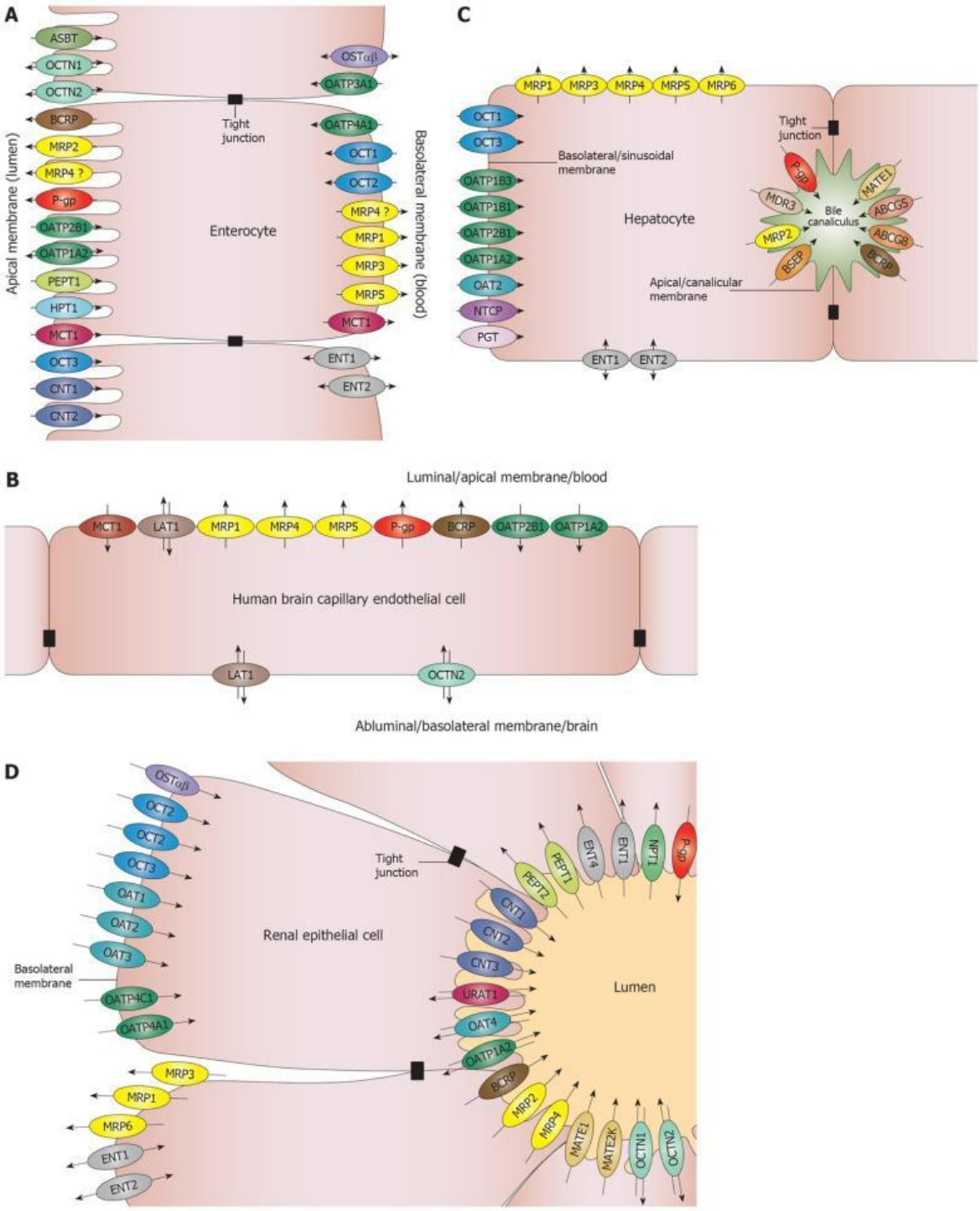

Figure 4: Expression of transporters in blood (A), brain (B), liver (C) and kidney (D) (adapted from (23))

Equilibrative Nucleoside Transporters (ENT)

ENTs can transport in both way and they are responsible of transport molecules down the concentration gradients. They are expressed in wide range, in lungs, heart, placenta, kidney, brain, ovaries and erythrocytes (20).

Multidrug and Toxin Extrusion Transporters (MATE)

MATEs belong to SLC family, even if they are efflux transporters. They are clarified to function as H^+/organic cation antiporter (20).

1.3. HISTAMINE

Histamine is an important biogenic amine, which plays a role as a local hormone a mediator of inflammation as well as neurotransmitter. Being a neurotransmitter it is involved in the sleep-wake process, memory, appetite, emotions, learning and also in pathological disorders such as Alzheimer's disorder, depression, epilepsy and schizophrenia (24).

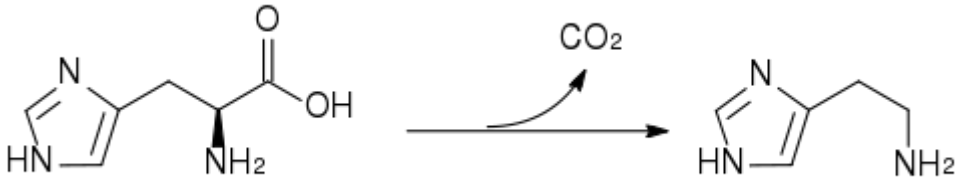

Figure 5: Histamine synthesis (adapted from: (25))

Histamine is produced in a single step from the decarboxylation of the amino acid histidine. The synthesis is carried out in the Golgi apparatus and can be stopped by α-fluoromethylhistidine (26). Later on, histamine is metabolized into methyl histamine by histamine methyltransferase, or into imidazoleacetaldehyde by diamine oxidase (27).

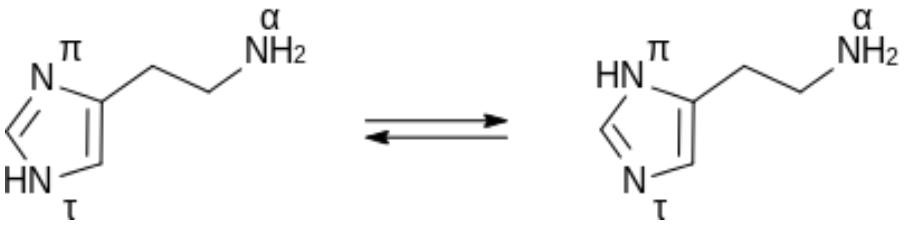

Figure 6: Histamine tautomers (adapted from: (28))

Histamine consists from imidazole and amine, the first having a pKa of 5.8 and the second having a pKa of 9.4. Being a double protonated molecule (Figure 6), present as a monocation or as a double cation, it cannot diffuse through the cell membrane, so it uses carrier-operated transport (24). Since a specific histamine transporter has not been discovered yet, protein OCT can be involved in this process.

It is still not certain who the best candidate is to transport histamine across cell membranes within the brain, because the expression of OCT1 and OCT2 in the brain is limited. Schneider and his co-workers discovered that OCT3 is responsible for the uptake of histamine by basophiles (29). In addition to that, it is also playing a significant role in the so-called ‘uptake 2’ of other monoamines such as dopamine, adrenaline, noradrenaline and serotonin (30).

1.4. QUERCETIN

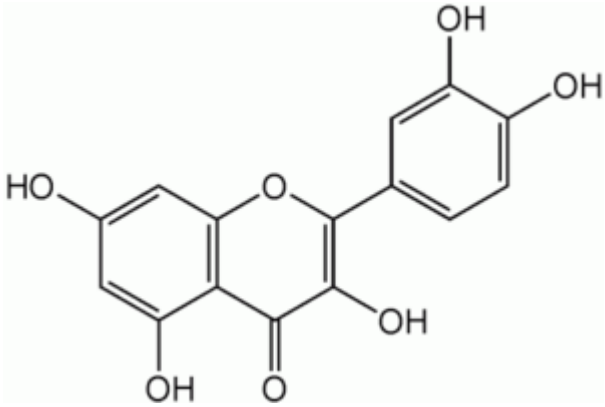

Figure 7: Structure of Quercetin (adapted from (31))

There are several subclasses of flavonoids: flavons, flavanones, flavones, isoflavones, anthocyanidins, and flavonols. Quercetin, a major representative of the flavonol subclass, is a very important bioflavonoid antioxidant, due to its ability to scavenge free radicals and bind transition metal ions, which allows quercetin to inhibit lipid peroxidation. Quercetin is usually found in red wine, fruits, tea and vegetable oils, and it is known for its multiple benefits in medicine due to its anti-inflammatory, anticarcinogenic and

antioxidant actions. It also has an inhibitory effect on lipid peroxidation, permeability in capillaries, and platelet aggregation (32) (33) (34).

After oral application, quercetin is in plasma found as sulfate or glucuronate conjugates as we see in Figure 8.

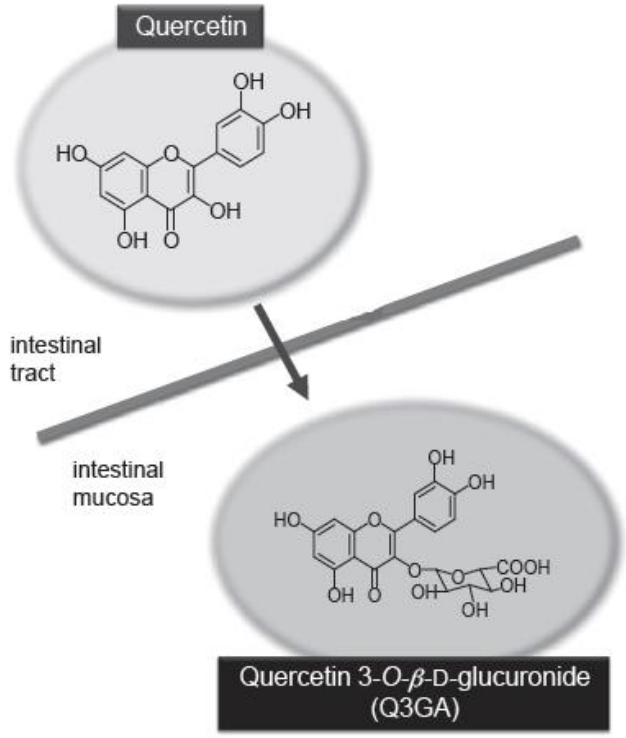

Figure 8: Metabolism of quercetin (adapted from (35))

Quercetin is a substrate for GLUT1 (human glucose transporter 1), which is expressed in the BBB and can facilitate the uptake of quercetin into the brain (36).

According on Table 3, quercetin inhibits multidrug resistance-associated protein 1/2 (MRP1/2), breast cancer resistance protein (BCRP) and it is potential inhibitor of OATP1A2, OATP2B1 and OAT1/3.

Table 3: presentation of molecules that inhibit or are substrates for certain transporters (adapted from (36)).

	P-gp	BCRP	MRP1	MRP2	MRP4	MRP5	OATP1A2	OATP 1B1	OATP 1B3	OATP 2B1
Phytochemicals										
Apigenin	?	I	I	I	?	?	I	?	?	?
Biochanin A	I[a]	I[a]	I[a]	?	?	?	?	I	?	?
Curcumin	I	I	I	?	?	I	?	?	?	?
Cyanidin	I	I	?	?	?	?	?	?	?	?
Daidzein	?	I[a]	I	?	?	?	?	?	?	?
EGCG	×	?	S	S	?	?	S, I	?	S	I
Epicatechin	?	?	S	S	?	?	S, I	?	S	I
Genistein	I[a]	S, I[a]	I	S	?	?	?	I	?	?
Narigenin	S, I	I	I	S	?	I	?	?	?	I
Naringin	I[a]	I	?	?	?	?	I	I	?	I
Puerarin	?	?	?	?	?	?	?	?	?	?
Quercetin	S, I	S,I	I	S, I	I	I	I	?	?	?
Resveratrol	I	S,I	I	?	I	×	?	?	?	?
Silymarin	I[a]	I	I	?	I	I	?	?	?	?

Quercetin is not soluble in water and its molecular mass (302 Da) places it in the range of lipohilic molecules that use the process of diffusion in order to get into the brain. But the problem is that, as it is shown in the structure on the Figure (9), quercetin is a negatively charged molecule.

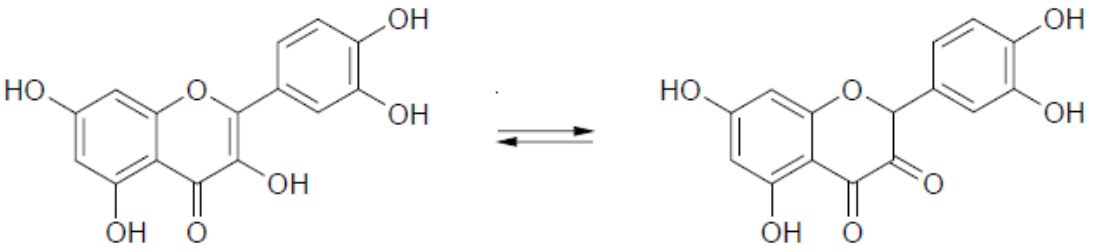

Figure 9: Tautomerisation of quercetin (adapted from (37))

It is still not known what kind of transporter the quercetin uses to pass across the BBB. The research about transporting flavonoids by bilitranslocase is a promising one, but for now, it is only limited to anthocyanins. A research was also made for quercetin; its results showed the possibility that quercetin is transported into the brain by bilitranslocase. Bilitranslocase is an organic anion carrier and it is a specific bilirubin membrane transporter, so it is usually found in liver. Since endothelial cells are also known to express bilitranslocase, it was an additional reason to explore this transporter in further details (38) (39) (40).

2. OVERALL AIM OF THE STUDY

The transport of substances through cell membranes as well as through the BBB depends on the physico-chemical properties of the substances. Water-soluble and charged substances diffuse through cell membranes using transporters. Since histamine and quercetin are both ions having a physiological pH, they cannot pass the cell membrane by the process of simple diffusion. Due to that, they use transporters to pass the cell membrane.

In this research, we will determine the basic characteristics of quercetin uptake into cultured neonatal rat astrocytes. Firstly, we will check the temperature and the concentration dependence of quercetin uptake. In the next step, we will evaluate sodium-, potassium-, chlorine- and ouabain-dependence. In the last step, we will compare quercetin and histamine uptake characteristics in neonatal rat astrocyte cultures.

In the graduate thesis, we will test the following hypotheses:

1. The transport through the cell membrane of either quercetin or histamine occurs via transporters.

2. Quercetin and histamine differ in their uptake characteristics.

3. MATERIALS AND METHODS

The materials used in the experimental part of the graduation thesis are listed in Table 4.

Table 4: Materials, used in the experimental part of the graduation thesis.

Material	Manufacturer
Gentamicin	Gibco, USA
Pyruvate	Gibco, USA
L-glutamine	Gibco, USA
DMEM	Gibco, USA
BSA	Sigma, USA
Leibovitz L-15 medium	Sigma, USA
HEPES	Sigma, USA
NaCl	Merck, Germany
KCl	Merck, Germany
KH_2PO_4	Merck, Germany
$MgSO_4$ x $7H_2O$	Sigma, USA
$CaCl_2$	Sigma, USA
Glucose	Merck, Germany
Quercetin	Sigma, USA
NaOH	Alkaloid, Macedonia
Aquasol	New England Nuclear, USA
Bio-Rad Protein Assay	Bio-Rad Laboratories, USA
Bilirubin	Sigma, USA
Trypsin-EDTA	Gibco, USA
Choline chloride	Gibco, USA
Ouabain	Sigma, USA
DMSO	Merck, Germany

3.1. Composition of solutions

Nutritive medium	
50 µl	Gentamicin
500 µl	Pyruvate
500 µl	L-glutamine
5 ml	Serum
50 ml	DMEM

Preparation medium	
50 mg	BSA
50 µl	Gentamicin
50 ml	L-15

Buffer A	
25 mM	HEPES
125 mM	NaCl
4.8 mM	KCl
1.2 mM	KH_2PO_4
1.2 mM	$MgSO_4x7H_2O$
1.7 mM	$CaCl_2$
5.6 mM	Glucose
pH	7.4

Buffer B	
25 mM	HEPES
125 mM	Choline chloride
4.8 mM	KCl
1.2 mM	KH_2PO_4
1.2 mM	$MgSO_4x7H_2O$
1.7 mM	$CaCl_2$
5.6 mM	Glucose
pH	7.2 – 7.4

Buffer C	
25 mM	HEPES
79.8 mM	NaCl
50 mM	KCl
1.2 mM	KH_2PO_4
1.2 mM	$MgSO_4x7H_2O$
1.7 mM	$CaCl_2$
5.6 mM	Glucose
pH	7.4

Buffer D	
25 mM	HEPES
125 mM	$Ca(NO_3)_2$ x $4H_2O$
4.8 mM	KCl
1.2 mM	KH_2PO_4
1.2 mM	$MgSO_4x7H_2O$
1.7 mM	$CaCl_2$
5.6 mM	Glucose
pH	7.2-7.4

3.2. METHODS

3.2.1. Experimental animals

For the preparation of primary cultures of astrocytes we used cerebral cortex of 3-4 days old neonatal Wistar rats (both sexes). All procedures on animals were used in accordance to Permission for Use of Laboratory Animals U34401-12/2013/3 issued by Veterinary Administration of Republic of Slovenia and according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Figure 10: Neonatal rats used in experiment. Picture was made with the permission of the Department of Pharmacology and Experimental Toxicology Faculty of Medicine.

3.2.2. Preparation of primary cell cultures of astrocytes

Neonatal rats were decapitated by a technical assistant who is certified to perform euthanasia of experimental animals. Assistant took brains out of the skull and immersed them in about 3-4 ml of separation medium L-15. Afterwards she removed meninges of cerebrum and dissected cerebrum from brain.

We immersed cortices in 4 ml of preparation medium and centrifuged it for 4 min at 1200 rpm. We washed minced cortical tissue with 3-4 ml of preparation medium L-15. Using pipettes we triturated the sediment and homogenized and washed it (4 min, 1200 rpm). The resulting suspension was transferred to Petri dish and passed through 20 gauge, 22 gauge and 25 gauge needle, sequentially. The cells were then passed through Nitex nylon mesh (pore diameter 0.75 μm).

Again we repeated centrifugation (4 min, 1200 rpm) and then we removed supernatant away. We added sediment to 15 ml of nutritive medium, mixed it and transferred the resulting suspension to the culture bottle(s) and we mixed it there again. We examined under the microscope in order to check if cells had been dissociated properly. We put the bottles in the incubator under the conditions: 37 °C in 95 % air and 5 % CO_2.

After 5 days we changed nutritive medium to cells and let them to grow till confluent. First we changed nutritive medium and after we put cells back to incubator at 37°C in 95% air and 5% CO_2. 6 hours later we transferred them to the shaker for 20 hours at 150 rpm at room temperature.

We repeated the process three times. After that we removed nutritive medium, washed cells with 10 ml DMEM and added 3-4 ml trypsin-EDTA. We put the bottles back to incubator for 20 min. When we put out bottles, we shook them really hard to detach cell. We examined the cells under microscope. We distributed the obtained suspension to more bottles, which provided the cells with more space for growth. We added additional nutritive medium (9-10 ml) and put them back to incubator overnight. Then we changed nutritive medium and due to that trypsin was removed. Cells were put back to incubator until next confluence.

We repeated the process with trypsin-EDTA and subcultured the cells into12 Petri dishes and cultured them until next confluence, when the cells were ready to perform the uptake experiments.

3.2.3. ^{3}H-quercetin uptake

3.2.3.1. The influence of temperature on ^{3}H-quercetin uptake

After having washed plates with 1 ml of buffer (A) containing $CaCl_2$ at 37 ºC, we incubated 540 µl of buffer A with $CaCl_2$ and 60 µl 10 nM ^{3}H-quercetin at 37 and 4 ºC for 30 min, 10 min, 5 min and 2 min. The reaction was stopped by placing the 12-well dish into ice-water bath and the cells were immediately afterwards washed 4-times with ice-cold buffer (A) without $CaCl_2$. The cells were lysed with 300 µl of 0.5 M NaOH.

250 µl of cell suspension was transferred to Eppendorf tubes and 1.5 ml of scintillation fluid Aquasol was added before we analysed radioactive decay in MicroBeta Scintillation Counter. The remained 50 µl was put to -20°C and used later on for protein determination. All experiments were made in triplicates.

The activity of 100 nM ^{3}H-quercetin in medium of incubation was 22.000.000 dpm.

3.2.3.2. The kinetics of ^{3}H-quercetin uptake

After having washed plates with 1 ml of buffer (A) containing $CaCl_2$ at 37ºC we incubated buffer A with $CaCl_2$ and 60 µl of increasing concentrations of ^{3}H-quercetin at 37 and at 4 ºC for 2 minutes. The concentration range of ^{3}H-quercetin spanned from the lowest to highest concentration. The reaction was stopped by placing the 12-well dish into ice-water bath and the cells were immediately afterwards washed 4-times with ice-cold buffer (A) without $CaCl_2$. The cells were lysed with 300 µl of 0.5 M NaOH.

The quantity of quercetin uptake into cells, were determined with liquid scintillation counter MicroBeta Trilux (Perkin Elmer, USA). The counter detects β-radiation of ^{3}H-Quercetin, when radio-labelled marker decays. The counter gives us a number of radioactive decays per minute (DPM) as a result.

We pippeted into eppendorf tubes 250 µL of our sample and we added 1.5 ml of Aquasol. Aquasol is universal solution for liquid scintillation counting and it consist aromatic

solvents and scintillators, the substances, which absorb energy of ionizing radiation and then emit light. Measurements were made at room temperature and pressure.

3.2.3.3. Na^+, K^+, Cl^- and ouabain-dependence

In order to determine Na^+- and Cl^--dependence on ^{3}H-quercetin uptake into astrocyte cells we replaced Na^+ in the uptake buffer (A) with choline chloride (Figure 12) and Cl^- with $Ca(NO_3)_2$ x $4H_2O$, respectively. We also conducted experiments in the presence of KCl in the uptake buffer (A) and Na^+K^+ATPase inhibitor ouabain (Figure11) in the uptake buffer (A). The preincubation of cells with a buffer of different composition lasted 30 minutes. After adding 60 µl 100 nM ^{3}H-quercetin, we incubated our samples for additional 2 minutes. The reaction was stopped as described in the previous section.

UB normal (A)	**UB with choline chloride (B)**	**UB with KCl (C)**	**UB normal (A) with ouabain**	**UB (D) with $Ca(NO_3)_2$ x $4H_2O$**

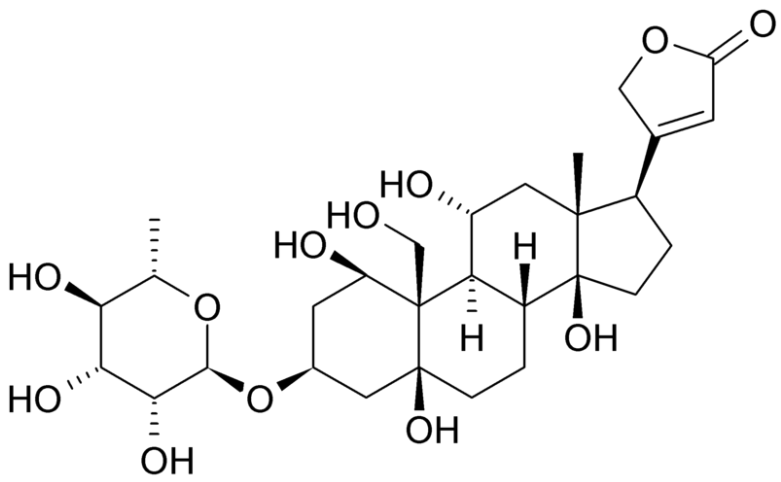

Figure 11: The structure of ouabain (adapted from ((41))

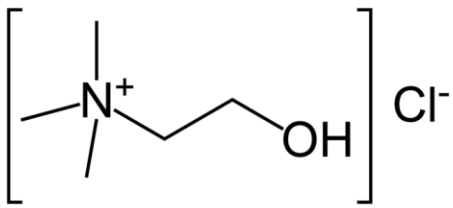

Figure 12: The structure of choline chloride (adapted from (42))

3.2.3.4. Bilirubin

In the last set of uptake experiments we determined the influence of bilirubin on ^{3}H-quercetin uptake. We washed the plates twice with 1 ml of uptake buffer (A) containing $CaCl_2$. We added 600 µl of buffer (A) with $CaCl_2$ (blanks) and 540 µl of buffer (A) with $CaCl_2$ and 60 µl of ^{3}H-quercetin (control); and 480 µl of buffer (A) with $CaCl_2$, 60 µl of ^{3}H-quercetin and 60 µl of bilirubin of different concentrations.

10^{-9} M	**10^{-8} M**	**10^{-7} M**	**5 x 10^{-7} M**	**7 x 10^{-7} M**	**10^{-6} M**	**10^{-5} M**

At first we preincubated buffer and bilirubin for 2 min at 37°C, then we added ^{3}H-quercetin and incubated for additional 2 min. Afterwards we stopped the reaction by placing the plates on ice and washing them four times with ice cold buffer (A) without $CaCl_2$, and repeated as described previously.

We perform the experiments in the dark because bilirubin (Figure 13) is very photosensitive.

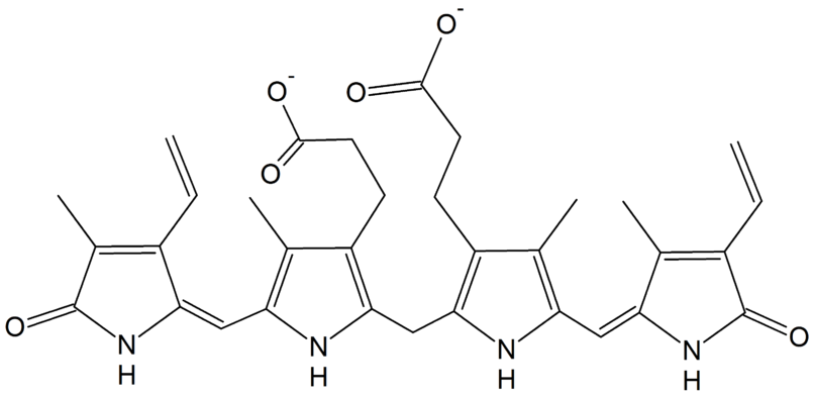

Figure 13: The structure of the bilirubin (adapted from (43))

3.2.4. Determination of protein concentration

For the preparation of the calibration curve we pipetted into test tubes increasing amounts of standard solution (1 µg/µl, Bio-Rad Protein Assay Standard) of proteins (2.5 µl, 5 µl, 8 µl, 10 µl, 20 µl, 30 µl, 50 µl). Then we diluted every solution with bi-distilled water to a total volume 800 µl, and in the end we added 200 µl of dyeing reagent.

We also prepared blind sample with same final volume 1000 µl, consisted of 800 µl of water and 200 µl of dyeing reagent. We mixed prepared solutions and left to react for 30 min, then we measured absorbance with spectrophotometer at a wavelength of 595 nm. We made two parallels for better results.

At same time we prepared our sample with 8 µl of proteins, 152 µl of water and 40 µl of dyeing reagent. We mixed prepared solutions and left to react for 30 min, then we measured absorbance with spectrophotometer at a wavelength of 595 nm. We made two parallels.

3.2.5. Data analysis

All experiments of uptake of ^{3}H-quercetin were made in triplicates or sometimes in quadriplicates and repeated at least twice. For data processing and graph drawings we used computer program GraphPad Prism, version 5.0 (GraphPad Software Inc., USA).

Results are shown as arithmetic mean ± standard error of the mean. We calculated a statistic difference between two samples with Student t-test. Statistically significant difference was $p \leq 0.05$.

4. RESULTS

Histamine and quercetin are both protonated molecules, due to that they cannot pass cell membrane or BBB with simple diffusion and they need transporter for passage either into the cell. The uptake of histamine into astrocytes has already been confirmed (30) (44), but a specific histamine transporter has not been elucidated, yet. Quercetin, shares some physico-chemical characteristic of bilirubin and due to that it might be a substrate for bilitranslocase.

Bilirubin is a lipophilic molecule with molecular mass of 548.67 Da (45), so it should pass the BBB, but normally we do not find large quantities of bilirubin in brain due to action of membrane transporter glycoprotein P-gp, which mediates efflux of bilirubin back to blood (46).

In the first part we examined the effect of temperature on quercetin uptake into cultured astrocytes. We performed the experiment at 37°C (physiological temperature) and 4°C. We used 4°C, because energy-driven processes (like ATP-mediated transport) do not appear at that temperature and because we do not yet know which compound is a specific inhibitor of quercetin transport.

4.1. Time dependence of quercetin and histamine uptake

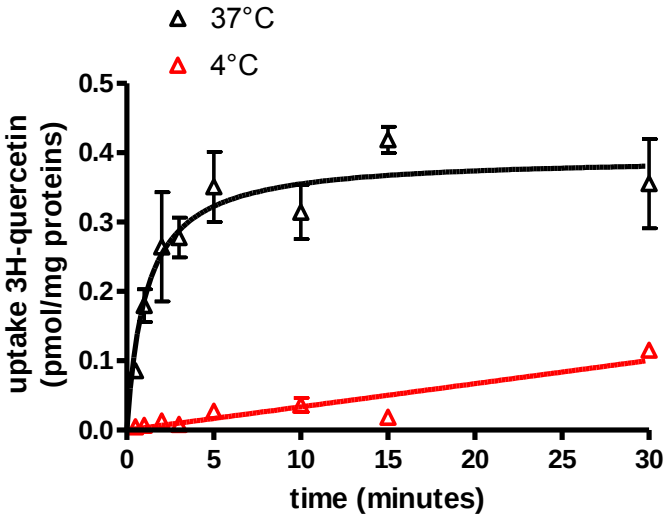

Figure 14: ^{3}H-quercetin uptake in neonatal rat astrocytes in dependence of time. Incubation with ^{3}H-quercetin (10 nM) was conducted for 2, 5, 10 and 30 minutes in buffer A. Results are shown as arithmetic mean ± standard error of arithmetic mean (n=6).

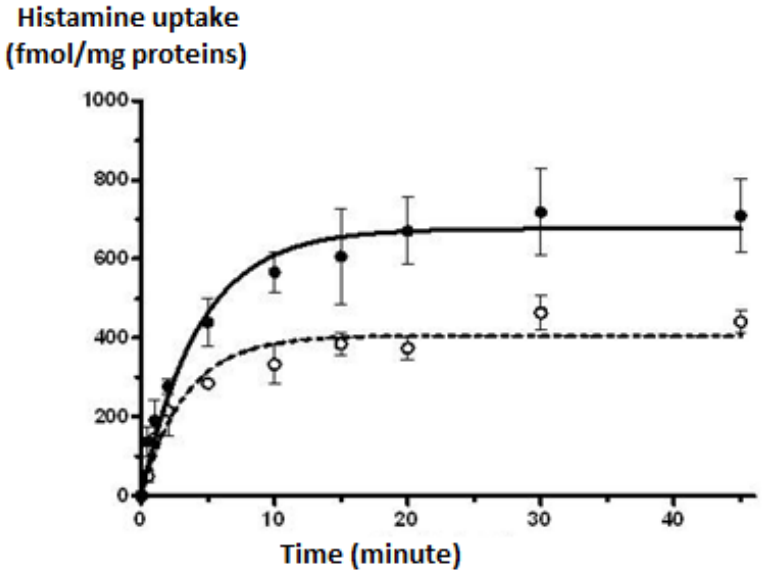

Figure 15: ^{3}H-Histamine uptake in neonatal rat astrocytes in dependence of time (adapted from (47))

37°C

In Figure 14 the time- dependence of ^{3}H-quercetin uptake is presented. The amount of quercetin taken up into cultured neonatal rat astrocytes increases in parallel mode with incubation time. The maximum of taken up quercetin appears at 5 minutes of incubation time. Afterwards, a plateau is reached. The maximal amount of quercetin taken up at 5 minutes of incubation is 0.323 pmol/mg protein, whereas at 15 minutes of incubation is 0.368 pmol/mg protein.

The time-dependent curves of either ^{3}H-quercetin or ^{3}H-histamine uptake are comparable. They are both saturable, but plateau of histamine uptake is achieved between 15-20 minutes of incubation.

4°C

Non-specific quercetin uptake (uptake occurring at 4°C) is linear and does not show a saturation, regardless of incubation time; whereas histamine uptake shows saturation.

4.2. Concentration dependence of ^{3}H-quercetin and ^{3}H-histamine uptake

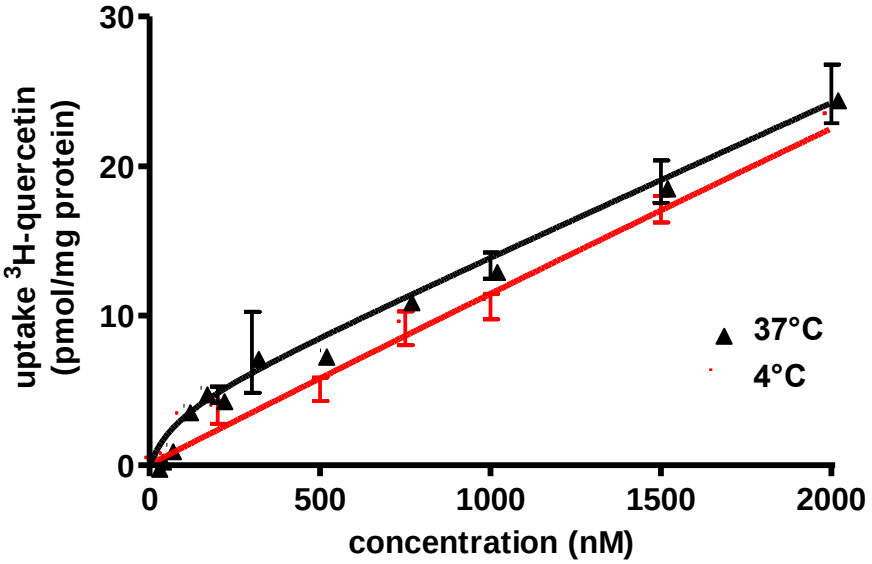

Figure 16: ^{3}H-quercetin uptake in neonatal rats astrocytes in dependence of concentration of ^{3}H-quercetin at pH=7.4. Analysis of kinetic K_{m1} of 176 nM and V_{max} 6 pmol/mg protein/min and K_{m2} 30.3 µM and V_{max2} 60 pmol/min), results are shown as arithmetic mean ± standard error of arithmetic mean (n=6).

Since there is no significant difference between the uptake of ^{3}H-quercetin at 37 °C and 4 °C, we did not calculate so called specific ^{3}H-quercetin uptake, presented on Figure 17.

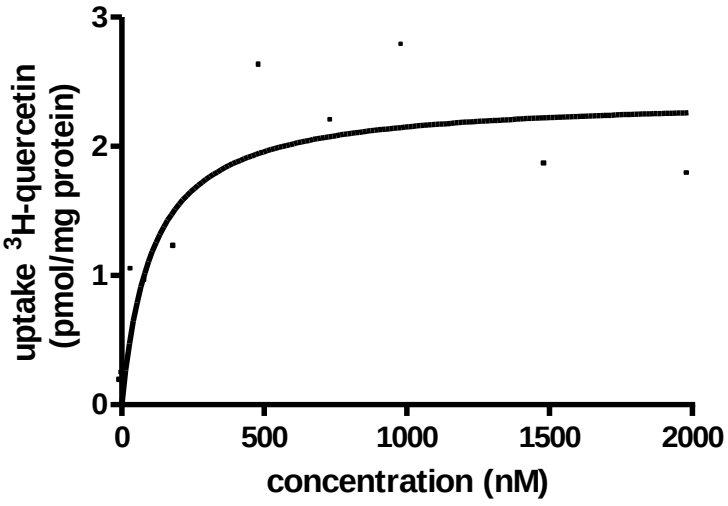

Figure 17: Presentation of calculated statistics and specific ^{3}H-quercetin uptake into neonatal rat astrocytes, determined as a difference between of ^{3}H-quercetin uptakes occurring at 4 °C and 37 °C in the graph above and shown in Figure 16.

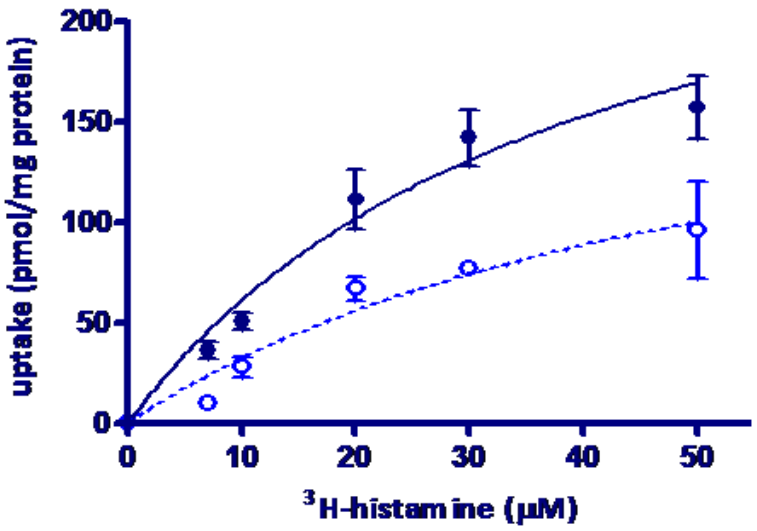

Figure 18: Concentration-dependent ^{3}H-histamine uptake in neonatal rats astrocytes of concentration dependence of ^{3}H-histamine at pH 7.4. First line presents the uptake at temperature 37°C, while the second line presents uptake at 4°C (Adapted from (48)). Kinetic parameter analysis of specific ^{3}H-histamine uptake revealed Km of 5.2 ± 2.1 M of and V_{max} of 11.2 ± 1.0 pmol/mg protein/min as determined by the best fit to $V = V_{max} . X / (K_m + X)$, where V is the rate of uptake, V_{max} – maximal rate of uptake, X – concentration of ^{3}H-histamine and K_m – affinity constant. Each point represents the mean ± SEM of six determinations.

Figure 16 shows the concentration-dependent uptake of quercetin into neonatal cultured rat astrocytes at 37°C (total uptake) and 4°C (nonspecific uptake). The curve representing uptake at 37°C has a small plateau appearing between 200 – 500 nM. But when incubating astrocytes with concentrations of quercetin higher than 500 nM quercetin, uptake began to increase again. These results might point to the fact that two transporters might be involved in quercetin uptake. The concentration-dependent quercetin uptake occurring at 4°C is represent by a line indicating that either the process is mediated by a simple diffusion or a carrier with very high capacity. The process of uptake in dependence of concentration is comparable for ^{3}H-quercetin and ^{3}H-histamin. The process is not saturated, what is seen in increasing lines at both graphs.

4.3. The effect of the bilirubin on the ^{3}H-quercetin uptake

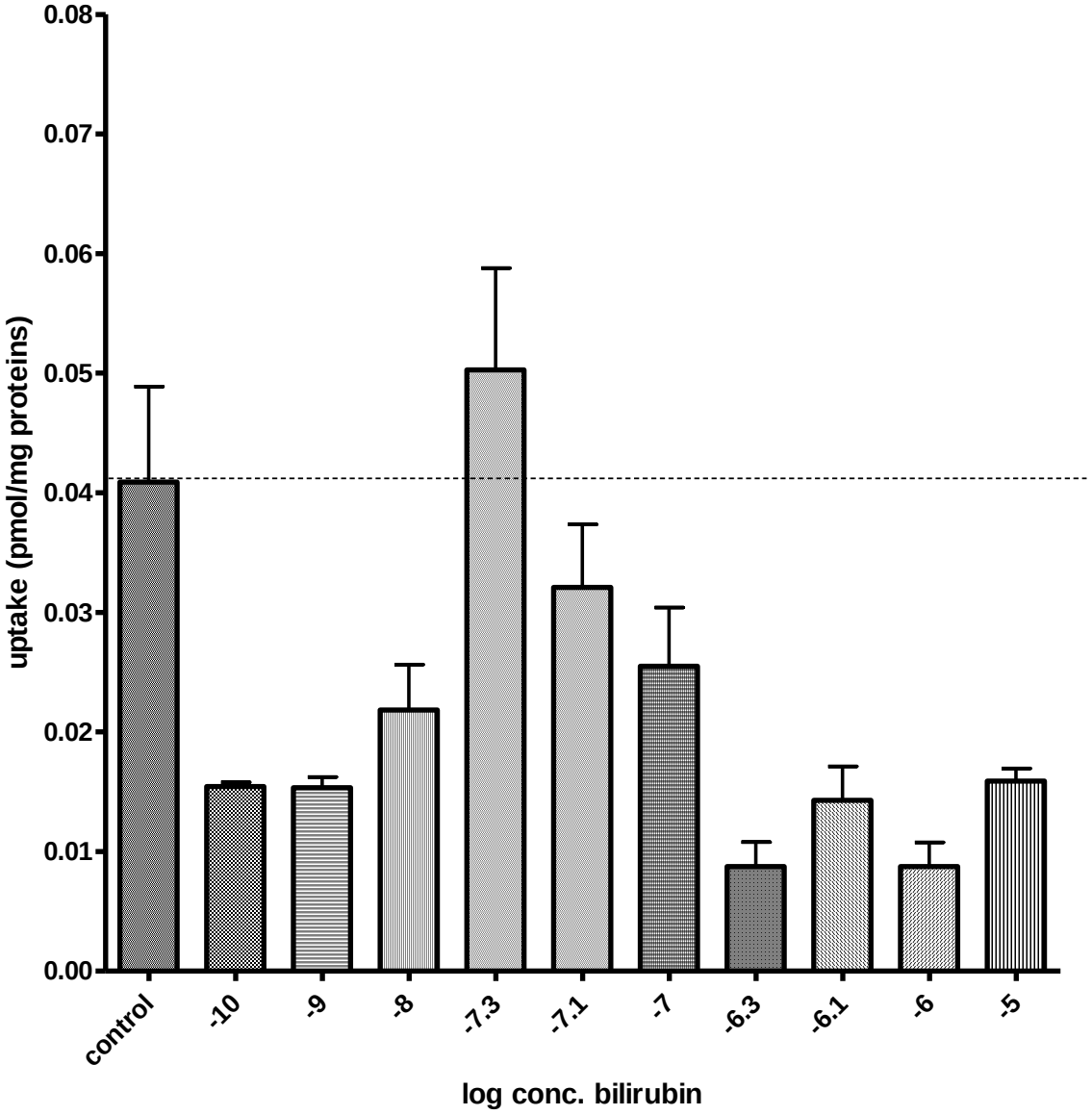

Figure 19: The effect of the different concentration of bilirubin on the ^{3}H-Quercetin uptake in neonatal rat astrocytes. Concentration is shown as log of concentration. Results are shown as arithmetic mean ± standard error of arithmetic mean (n=6), *p<0.05.

We preincubated cultured astrocytes for 2 minutes with different concentrations of bilirubin (concentration range from 0.1 nM – 10 μM). Then ^{3}H-quercetin was added and cells were incubated for additional 2 minutes when the reaction was stopped.

As it is seen from Figure 19, bilirubin inhibits ^{3}H-quercetin uptake in a biphasic way. Very low concentrations (below 50 nM) significantly inhibit ^{3}H- quercetin uptake. 70 nM bilirubin has no effect on quercetin uptake, whereas higher concentrations inhibit quercetin uptake in a concentration dependent manner reaching a plateau when micromolar and higher concentrations of bilirubin used. The results of inhibition uptake studies show that quercetin is carried by at least two different transport molecules across cell membrane of astrocytes.

Results in Figure 19 are shown as log of concentration for better presentation.

The maximum ^{3}H-quercetin uptake is observed at concentration of 50 nM, while at the other concentration of bilirubin is the ^{3}H-quercetin uptake lower than a control.

4.4. The effect of Na+, Cl- , K+ and Na+K-ATPase activity on ^{3}H-quercetin uptake

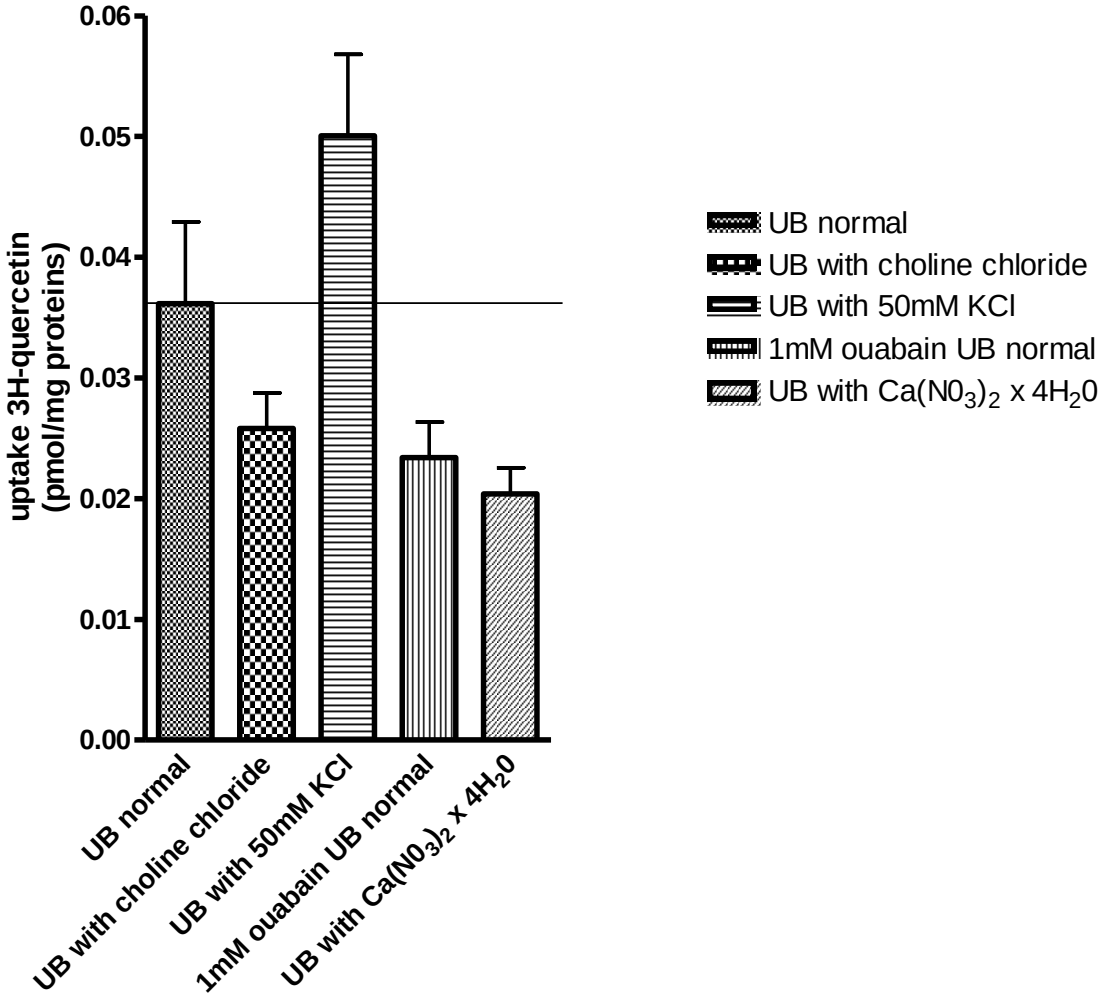

Figure 20: The effect of different substrates on the ^{3}H-Quercetin uptake in neonatal rat astrocytes. Results are shown as arithmetic mean ± standard error of arithmetic mean (n= 6), ***p<0.001.

The cells were transferred to different uptake buffer 30 minute before ^{3}H-quercetin was added. After that cells were incubated for 2 minutes before reaction was stopped.

Observing sodium-, chloride, potassium and ouabain-dependence we checked whether transport of quercetin is mediated via co-transporter or simporter. Driving force of some co-transporters is mediated by difference in ion gradients across cell membrane, eg sodium, chloride, as well as ATP-dependent. As we can see from Figure 20, the transport of quercetin is not sodium-dependent, because the uptake of quercetin in buffer in which sodium salts were replaced with choline chloride did not significantly changed quercetin uptake. The excess of potassium chloride increased quercetin uptake into cultured astrocytes, whereas replacement of chloride salts with calcium nitrate or inhibition of Na+K+-ATPase with ouabain significantly decreased quercetin uptake into cultured astrocytes. The results indicate that at least two processes are involved in quercetin uptake, an active ATP-dependent and Cl- dependent process, and a passive quercetin-concentration dependent process (uptake2- like process).

5. DISCUSSION

The supply of the brain with various molecules, such as nutrients and medicines, presents an interesting topic that has been increasingly investigated by scientists. The entrance of different molecules to the brain occurs via highly regulated processes. Various molecules enter the brain through the BBB by means of active-transported or facilitated diffusion, and rarely by means of a simple diffusion, before binding to specific receptors and causing different effects. The BBB is a highly selective permeability barrier which allows the passive passage of lipophilic molecules or molecules with a relatively small molecular mass (like water or ethanol), whereas glucose and amino acids pass the blood brain barrier using different transporter(s). The kind of transport molecules use to pass through the BBB mostly depends on physico-chemical characteristics of the molecule in question (medicine, nutrient), such as solubility, size and charge. The presence and the function of different transporters within the BBB play a significant role in the availability of different drugs within the central nervous system (e.g. aromatic amino acid transporter carries levodopa into the brain; a lot of neuroleptic and antiepileptic drugs are substrates for p-glycoprotein, an efflux transporter). The transporters are not present on the BBB, but can regulate the passage of different molecules through cell membranes.

In the present research work, we focused on the comparison of the uptake characteristics of two different molecules, histamine and quercetin. Histamine is an important neurotransmitter within mammalian brain, as well as a local hormone having an important role as a mediator of allergic reactions. Histamine is mostly present as a monocation having a physiological value of pH. Quercetin is an important flavonoid with antioxidant actions, also acting as an inhibitor of histamine release. Several groups of researchers have already investigated the kinetics, the characteristics and the role of histamine uptake; however, since the literature data on quercetin uptake is scarce, we do not know much about the way quercetin transports into cells.

There we two reasons to compare the uptake of quercetin with the uptake of histamine:

1. Since there are no data on quercetin uptake in the scientific literature, it makes it easier to compare the results of quercetin uptake with the results of histamine

transport, because the transport characteristics of histamine had been intensively studied in our laboratory.

2. The other interesting feature of quercetin is its ability of blocking histamine release from mass cell and basophiles and his capability of acting as an "antihistamine".

Our hypothesis that histamine and quercetin differ in the kinetic characteristics of the transport was confirmed. Both uptakes (histamine and quercetin's) are time-dependent, but the uptake of quercetin is significantly faster (reaching plateau at 5 minutes of incubation) than histamine transport (reaching plateau between 15-20 minutes of incubation).

Further on, results also showed that histamine uptake depends on the concentration of radiolabelled histamine (44). We found that the transport of quercetin is also a concentration-dependent process. Concentration-dependent histamine uptake is a saturable process, whereas quercetin uptake did not show saturation, in spite of the fact that micromolar concentrations of quercetin were used in the experiment. It means that the capacity of quercetin uptake (60 pmol/mg protein/min) should be considerably greater than the capacity of histamine uptake (11.2 pmol/mg protein/min). In addition to greater capacity, in cultured neonatal astrocytes, quercetin should be taken up by two different processes (6 pmol/mg protein/min and 60 pmol/mg protein/min) involving multiple transporters.

Specific uptake is usually calculated as the difference between total and non-specific uptake. Non-specific uptake is the uptake of observed compound, which occurs when the transporter is blocked by a specific uptake inhibitor (e.g. fluoxetine is inhibitor of serotonin transporter). Since neither specific histamine transporter nor specific quercetin transporter inhibitors are known, we used the uptake occurring at 4ºC as the non-specific uptake. At 4ºC, no ATP-driven process occurs. The only uptake occurring at 4ºC is either a passive diffusion or concentration gradient-driven facilitated diffusion. If we compare the uptakes shown on Figure 16 and 18, we see that the majority of uptake of quercetin occurs by process or processes that do not require ATP, whereas half of the observed histamine uptake occurs by a process that is energy-dependent, and the other half of histamine uptake resembles the so-called "uptake 2" (uptake occurring via a non-selective transporter like OCT).

Regarding their molecular weight, histamine and quercetin are both small molecules, but due to their structure, they carry a charge at physiological value of pH, so they cannot freely pass either the BBB or the cell membrane with a simple diffusion but have to have the help of transporters.

The specific transporter for histamine has not been identified yet, but a lot of research groups propose that the most possible transporter that carries histamine across cell membranes could be OCT3, the member of the SLC superfamily.

The uptake of quercetin has not been investigated in details yet. The first group (Sabina Passamonti's group) that studied quercetin obtained some promising results, pointing out that bilitranslocase, a specific bilirubin membrane transporter, can also carry quercetin across cell membranes (40). To establish whether bilitranslocase is responsible for the uptake of quercetin into astrocytes, we could perform the uptake experiment after pre-incubation of astrocytes with the same antibodies that Sabina Passomonti's group had used.

Since the concentration-uptake curve of quercetin indicates that there are two transporters involved in the uptake process, we are suggesting that a representative of OAT group or a transporter that carries nucleotides can play a role therein.

OATs are expressed within the brain and carry organic anions across cell membranes. The transport using OAT is not an ATP-driven process; it is a facilitated diffusion using concentration gradient of solute as a driving force.

We assume that quercetin might transport through astrocyte cell membranes with bilitranslocase and one of OATs, since they are sharing some similar physico-chemical characteristics with bilirubin. Bilirubin can act as a substrate for both bilitranslocase and OATs.

We can confirm this claim with the help of our results, which showed very low concentrations of bilirubin, below 50 nM, and concentrations above 70 nM for inhibited

quercetin uptake in neonatal rat astrocytes. The shape of the curve depicting the effect of bilirubin on quercetin uptake indicates the possibility of existence of two transporters involved in the quercetin uptake in astrocyte membranes. These results are in accordance with the results of inhibited quercetin uptake into endothelial cells, depicted in the study by A. Maestro and colleagues (40).

With sodium-, chloride-, potassium- and ouabain-dependence, we verified if the transport of quercetin is mediated via co-transporters, since the aforementioned ions, together with Na+ K+- ATPase, play an important role in transporter function.
We replaced the sodium salt with choline chloride, but results showed a decrease in quercetin uptake. However, the decrease not being statistically significant, we cannot say that quercetin uptake changes are sodium-dependent. The excess of potassium increases quercetin uptake as well, but again, the increase is statistically insignificant. However, we did find out that quercetin uptake is chlorine-dependent. Accordingly, we assumed that quercetin is not sodium-dependent or potassium-dependent, since Na+- and K+ dependence is characteristic for ATP-driven active transport.

Ouabain is a cardiac glycoside known for its inhibition of Na+/K+- ATPase. A blockage of Na+/K+- ATPase results in the disability of active transport. Quercetin uptake is partially and not in a statistically significant manner dependent on the activity of Na+K ATPase, which is in accordance with the presupposition that bilitranslocase and OATP can carry quercetin across astrocyte cell membrane.

If we compare the quercetin uptake with the transport of histamine into neonatal rat astrocytes, histamine uptake is only Na+-dependent (24, 51) and ouabain-sensitive (51), but not chloride-dependent. In addition to that, the kinetics of histamine uptake depends on the brain region. We have not evaluated that for quercetin, because the present study is the first study of the kind, but also because we were studying quercetin uptake in astrocytes derived from the brain cortex.

To sum up the above, quercetin cannot enter the cell by manner of simple diffusion but must use a transporter, which can be either bilitranslocase or a representative of OAT. The transport of quercetine is a rapid, C1-dependent and not ATP-driven process.

6. CONCLUSSION

The results obtained by our research prove that there are some differences in histamine and quercetin uptake in neonatal rat astrocytes.

Both hypotheses have been confirmed by our study.

1. Transport of the histamine and quercetin through the cell membrane occurs via transporters. Histamine is using cation carriers to pass the astrocyte cell membrane, while quercetin might use anion carrier SLC-OAT or bilitranslocase. The results show the impact of bilirubin on the quercetin uptake.

2. The kinetic characteristics of transport are different for quercetin and histamine. Histamine and quercetin uptakes depend on the time, temperature and concentration. The process of histamine uptake is saturated, while the process of quercetin uptake is only saturated at 37°C in dependence of time.

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