Transporters involved in adult rat cortical astrocyte dopamine uptake: kinetics, expression and pharmacological modulation

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Abstract

Astrocytes, glial cells in the central nervous system, perform a multitude of homeostatic functions and are in constant bidirectional communication with neuronal cells, a concept named the tripartite synapse, however their role in the dopamine homeostasis remains unexplored. The aim of this study was to clarify the pharmacological and molecular characteristics of dopamine transport in cultured cortical astrocytes of adult rats. In addition, we were interested in the expression of mRNA of dopamine transporters as well as dopamine receptors D1 and D2 and in the effect of dopaminergic drugs on the expression of these transporters and receptors. We have found that astrocytes possess both Na^+-dependent and Na^+-independent transporters. Uptake of radiolabelled dopamine was time-, temperature- and concentration-dependent and was inhibited by decynium-22, a plasma membrane monoamine transporter inhibitor, tricyclic antidepressants desipramine and nortriptyline, both inhibitors of the norepinephrine transporter. Results of transporter mRNA expression indicate that the main transporters involved in cortical astrocyte dopamine uptake are the norepinephrine transporter and plasma membrane monoamine transporter. Both dopamine receptor subtypes were identified in cortical astrocyte cultures. 24-hour treatment of astrocyte cultures with apomorphine, a D1/D2 agonist, induced upregulation of D1 receptor, norepinephrine transporter and plasma membrane monoamine transporter, whereas the latter was downregulated by haloperidol and L-DOPA. Astrocytes take up dopamine by multiple transporters and express dopamine receptors, which are sensitive to dopaminergic drugs. The findings of this study could open a promising area of research for the fine-tuning of existing therapeutic strategies.

Transporters involved in adult rat cortical astrocyte dopamine uptake: kinetics, expression and pharmacological modulation

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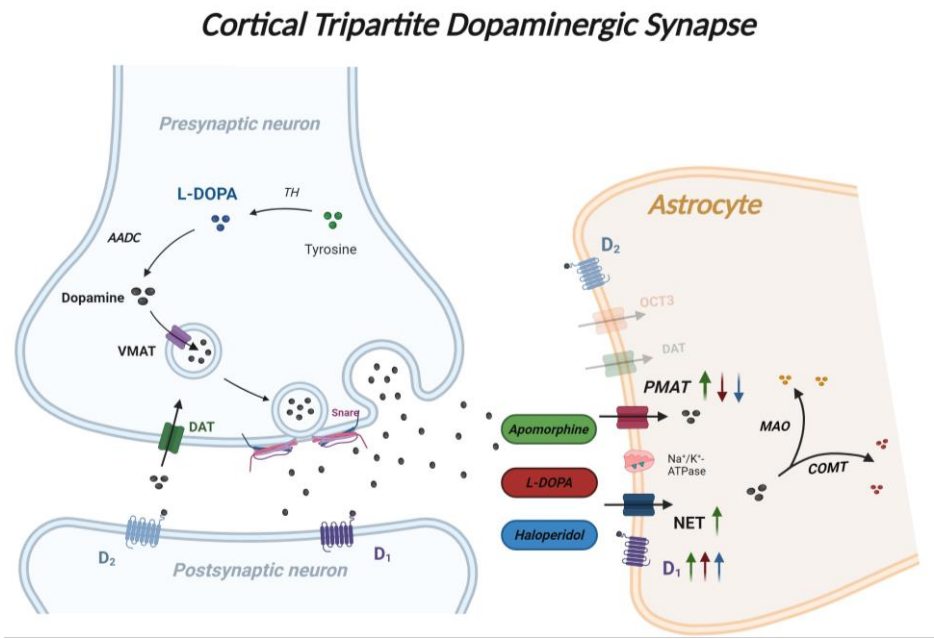
Key words: dopamine, NET, PMAT, dopamine uptake, cortical astrocytes, adult rat

Graphical Abstract



Tripartite dopaminergic synapse (created with Biorender).

TH – tyrosine hydroxylase, AADC - aromatic l-amino acid decarboxylase, VMAT – vesicular monoamine transporter, MAO – monoamine oxidase, COMT – catechol-O-methyltransferase, PMAT – plasma membrane monoamine transporter, NET – norepinephrine transporter, DAT – dopamine transporter, OCT3 – organic cation transporter 3

Abstract

Astrocytes, glial cells in the central nervous system, perform a multitude of homeostatic functions and are in constant bidirectional communication with neuronal cells, a concept named the tripartite synapse, however their role in the dopamine homeostasis remains unexplored. The aim of this study was to clarify the pharmacological and molecular characteristics of dopamine transport in cultured cortical astrocytes of adult rats. In addition, we were interested in the expression of mRNA of dopamine transporters as well as dopamine receptors D1 and D2 and in the effect of dopaminergic drugs on the expression of these transporters and receptors. We have found that astrocytes possess both Na^+-dependent and Na^+-independent transporters. Uptake of radiolabelled dopamine was time-, temperature- and concentration-dependent and was inhibited by decynium-22, a plasma membrane monoamine transporter inhibitor, tricyclic antidepressants desipramine and nortriptyline, both inhibitors of the norepinephrine transporter. Results of transporter mRNA expression indicate that the main transporters involved in cortical astrocyte dopamine uptake are the norepinephrine transporter and plasma membrane monoamine transporter. Both dopamine receptor subtypes were identified in cortical astrocyte cultures. 24-hour treatment of astrocyte cultures with apomorphine, a D1/D2 agonist, induced upregulation of D1 receptor, norepinephrine transporter and plasma membrane monoamine transporter, whereas the latter was downregulated by haloperidol and L-DOPA. Astrocytes take up dopamine by multiple transporters and express dopamine receptors, which are sensitive to dopaminergic drugs. The findings of this study could open a promising area of research for the fine-tuning of existing therapeutic strategies.

1. Introduction

Astrocytes, the most abundant cell type in the central nervous system (CNS) [1-3], play a vital role in maintaining CNS homeostasis and are actively involved in various functions. They are crucial for synapse formation and regulation, influencing synaptic connectivity, plasticity, and responding to pathological insults [4-7]. Despite lacking the ability to generate action potentials, astrocytes engage in constant bidirectional communication with neurons, forming what is known as the tripartite synapse [8-11]. Through fluctuations in intracellular second messengers such as Ca^{2+} and the release of gliotransmitters, astrocytes respond to synaptic activity and contribute to the regulation of synaptic transmission and plasticity [8,12]. While their involvement in glutamate homeostasis is well-studied [13,14], their specific role in the homeostasis of neurotransmitter dopamine (DA), remains less explored.

DA plays a vital role in various processes including spatial memory formation, motivation, arousal, reward, pleasure, lactation, sexual behaviour, nausea, and motor function [15]. Clearance of DA from the extracellular space back into the presynaptic neuronal cells occurs via two types of transport mechanisms. High-affinity, low-capacity uptake 1 transport and slower, low-affinity, high-capacity uptake 2 transport [16-18]. Uptake 1 transport of DA primarily relies on the dopamine transporter (DAT) [19] or norepinephrine transporter (NET), depending on the brain region and transporter availability. In certain brain regions with sparse DAT distribution, such as the cortex, NET has been found to have equal affinity for DA and plays a prominent role in DA uptake, especially in conditions like Parkinson's disease [17,20]. Both uptake 1 transporters share structural similarities, with similar amino acid sequences [21], and their functionality depends on the Na^+/Cl^- ion gradient provided by the Na^+/K^+ ATPase [16-18,22]. These transporters are important targets for drugs used in the treatment of various neuropsychiatric disorders [18]. In addition to the uptake 1 transporters, DA transport is also mediated by uptake 2 transporters, capable of transporting multiple organic cations, namely organic cation transporters OCT1, OCT2, OCT3 [23-25] and the plasma membrane monoamine transporter (PMAT) [26-29]. The coexistence of both low and high affinity neurotransmitter transporters enables rapid adjustments of monoamine neurotransmitter concentrations based on location and function. In the presence of drugs inhibiting high-affinity transporters, the role of low-affinity transporters becomes crucial for the reuptake of neurotransmitters [30].

Astrocytes express various transporters involved in DA uptake and storage, including the OCTs [24,31-34], vesicular monoamine transporter 2 (VMAT2) crucial for DA storage [35], by some studies DAT [36,37], NET [38,39], and PMAT [35,40-42]. However, the exact kinetics and transporter specificity of DA uptake into astrocytes are not well-established. Some studies suggest that astrocytic DA uptake is Na^+-independent [43], while others propose that it is Na^+-dependent and mediated by NET [44,45]. Astrocytes also express the majority of DA metabolizing enzymes, such as monoamine oxidase-B (MAO-B) and catechol-O-methyltransferase (COMT), suggesting that astrocytes may contribute to the metabolism of DA after uptake [17,35]. Additionally, astrocytes express the neutral amino acid transporter LAT involved in the transport of the synthetic DA precursor L-DOPA and the enzyme aromatic amino acid decarboxylase (AADC), essential for DA synthesis from L-DOPA [46,47], however the production of DA from L-DOPA in astrocytes appears to be limited [36,48].

DA exerts its effects by binding to two types of DA receptors (DR). D1-type receptors (DRD1 and DRD5) are excitatory, while D2-type receptors (DRD2, DRD3, DRD4) are inhibitory [16,49]. Astrocytes have been reported to express various DA receptors, but their expression pattern can vary across studies [50-52]. Activation of DA receptors in astrocytes can lead to changes in intracellular Ca^{2+} levels and other signalling molecules, such as cAMP, with effects that require further investigation [53,54]. Emerging evidence suggests that astrocyte DA receptors respond to drugs used in the treatment of

neuropsychiatric disorders, such as Parkinson's disease and schizophrenia, by increased astrocyte DA clearance in Parkinson's disease, uptake and accumulation of L-DOPA in rat astrocytes, and changes in intracellular signalling pathways in rat astrocytes after exposure to DA agonists and antipsychotic medications [16,36,51].

According to our knowledge, this is the first study to use to use primary astrocyte cultures prepared from normal adult rats as a model system to study DA uptake. The aim of our study was to investigate DA uptake into cultured cortical adult rat astrocytes, elucidate the kinetic properties of DA transport, and identify the specific transporters involved. In addition to that, we explored, whether treatment with drugs used to combat diseases such as Parkinson’s disease, schizophrenia, and depression might induce changes in the expression of transporters and receptors involved in astrocyte DA homeostasis. Understanding the role of astrocytes in the dopaminergic synapse may provide valuable insights into the pathogenesis of CNS disorders and potentially uncover new therapeutic strategies.

2. Materials and Methods

2.1. Materials

All cell culture reagents, except fetal bovine serum (FBS, Cambrex IEP GmbH (Wiesbaden, Germany)) were obtained from Gibco, Invitrogen (Paisley, Scotland, UK). [^{3}H]-dopamine (2220 GBq/mmol) was purchased from Perkin Elmer (Massachusetts, USA), E.Z.N.A.® Total RNA Kit I from Omega Bio-Tek (Norcross, GA, USA), High Capacity cDNA Reverse Transcription Kit, TaqMan Gene Expression Assays and TaqMan® Universal PCR Master Mix from Applied Biosystems (Carlsbad, CA, USA). Decynium-22 (D22), nortriptyline HCl, apomorphine HCl, haloperidol, L-deprenyl HCl and tropolone were obtained from Sigma Aldrich (St. Louis, MO, USA), desipramine HCl and amitriptyline HCl were obtained from Sandoz (Cham, Switzerland). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was obtained from Promega (Madison, WI, USA).

2.2. Methods

2.2.1. Animals

For the purpose of our study, we have obtained the permission of the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection issue U34401-20/2017/2. This study was approved by the National Veterinary Administration (approval numbers U34401-23/2022/6, approval date 20.06.2017 and U34401-23/2022/6, approval date 23.12.2022). We used brain from redundant sexually mature animals, weighing 180 – 200 g of both genders, and all procedures complied with the relevant Slovenian and European legislation.

2.2.2. Primary astrocyte cultures

We obtained cortical and kidney tissue from adult rats, species *Rattus norvegicus*, breed Wistar. Cultured rat cortical astrocytes were prepared from adult rat cortices by a well-established protocol, routinely used in our laboratory [55]. Astrocyte cultures were grown and maintained in T-75 flasks (Falcon, Corning, USA) and 12-well plates (Falcon). Briefly, cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS, 1 mM pyruvate, 2 mM glutamine, and 25 µg/mL gentamycin/streptomycin in humidified 95% air–5% CO_2 atmosphere. Confluent cultures were shaken at 225 RPM overnight, the medium was changed the next morning and this process was repeated a total of three times. After the third overnight shaking, the cells were trypsinized, washed in DMEM and subcultured into 12-well plates, the medium was changed each week from then on. After three weeks, the cultures contained 93–100% type 1 astrocytes.

2.2.3. [^{3}H] – dopamine uptake experiments

(i) Temperature, time and concentration dependence of [^{3}H]-DA uptake

Monolayer cultures in 12-well plates were washed twice in the uptake buffer containing $CaCl_2$ (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.4 mM $CaCl_2$, and 5.6 mM glucose, pH 7.4) and MAO (tropolone, 0.1 mM) and COMT (L-deprenyl, 0.1mM) inhibitors at 37 °C (total uptake) and at 4 °C (non-specific uptake). The uptake buffer was added to each well in addition to [^{3}H]-DA, resulting in a total volume of 500 µL. For the time course (0 – 60 min) curve, transport was initialized by the addition of radiolabelled dopamine and stopped by placing the plates into an ice-water bath at different time points. The buffer was removed and the plates were washed four times with ice-cold uptake buffer without Ca^{2+}. The cells were lysed in 300 µL of 0.5 M NaOH. 250 µL of each sample was transferred to a scintillation vial and the radioactivity was measured. From 4 µL of each sample we measured the protein concentration using the Bradford method using the Bio-Rad Protein Assay

(Hercules, CA, USA). The design of the experiments was the same for the concentration dependence curve, where the experiments were performed with the [^{3}H]-DA concentration ranging from 0.03 to 750 µM and the reaction was stopped after 15 min for each tested concentration.

(ii) Inhibition by antidepressants and decynium-22 and ouabain sensitivity of [^{3}H]-DA uptake

For the inhibition studies, monolayer cultures in 12-well plates were prepared in the same manner as described previously and preincubated in different test compounds at various concentrations (10^{-8} – 10^{-3} M) for 20 minutes, then incubated with the [^{3}H]-DA concentration of 30 nM for 15 minutes. The chosen test compounds were desipramine, nortriptyline and amitriptyline and non-specific uptake transporter inhibitor decynium-22 (D22). The reaction was stopped by placing the plates on ice and washing with ice-cold uptake buffer without Ca^{2+} in the same manner as described above. The cells were lysed by the addition of 0.5 M NaOH, samples collected and DA uptake determined in the same manner as described above. Ouabain sensitivity was tested by a 20-minute preincubation of cell plates with 0.1 mM and 1 mM ouabain and then carried out in the same manner as described above.

2.2.4. Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from adult rat cortical astrocyte cell cultures, as well as cortical and kidney tissue samples using the E.Z.N.A.® Total RNA Kit I. 1 µg of RNA was used to synthesize cDNA utilizing High-Capacity cDNA Reverse Transcription Kit, according to manufacturer's instructions. qPCR was performed using TaqMan™ Universal PCR Master Mix and TaqMan Assays: SLC6A3 (Rn00562224_m1), SLC6A2 (Rn00580207_m1), SLC22A1 (Rn00562250_m1), SLC22A2 (Rn00580893_m1), SLC22A3 (Rn00570264_m1), SLC29A4 (Rn01453824_m1), DRD1 (Rn03062203_s1), DRD2 (Rn00561126_m1) and ß-actin (Rn00667869_m1), according to the manufacturer's instructions, in QuantStudio™ 3 System. Expression of target genes was normalized to expression of ß-actin according to the equation [target/reference] = [$EFF_{reference}$^$Cq_{reference}$]/[EFF_{target}^Cq_{target}], where Cq is quantification cycle and EFF is amplification efficiency (expressed as a value between 1 and 2). EFF was determined with LinRegPCR software [56].

2.2.5. Cell treatments

Confluent three weeks old cortical adult rat astrocyte cell cultures plated in 12–well plates were treated with three different compounds, haloperidol, apomorphine and L-DOPA for 24 hours. The concentration of drugs used for the cell treatment was set at 100 µM, based on cell viability experiments. The viability of astrocytes, plated in 96-well plates, was examined after 24-hour treatment with the drugs at concentrations up to 150 µM, using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS).

2.2.6. Data analysis

The uptake experiments were routinely carried out in triplicates and each experiment was repeated at least twice. The kinetic parameters (such as K_m and V_{max}) were obtained using GraphPad Prism 9.5. software (San Diego, USA). Comparison among data was carried out using either t-test or ANOVA. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Time and temperature dependence of [^{3}H]-DA uptake into cultured adult rat astrocytes

Astrocyte cultures were prepared from cortices of adult Wistar rats as previously described [55,57]. Basic characteristics of DA transport (time and concentration dependence) were determined first, since phenotypic properties of cultured astrocytes can be affected by the method of preparation, culture conditions and animal species used [58]. Astrocytes were incubated with 30 nM [^{3}H]-DA and the characteristics of time and temperature dependence were assessed at 37 °C, determined as total DA uptake, and at 4 °C, determined as non-specific uptake, based on previous studies on cultured rat astrocytes [34]. Cortical astrocytes were exposed to 30 nM concentration of [^{3}H]-DA for a maximal time span of 60 min. [^{3}H]-DA uptake reached a plateau at 20 min (Fig. 1). Based on these experiments the incubation time of DA uptake was set to 15 minutes.

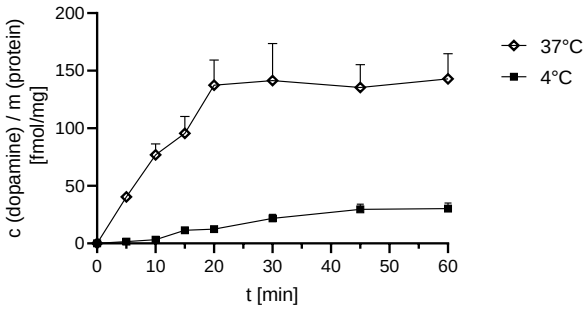


Figure 1 Time dependence of [^{3}H]-DA uptake into cortical adult rat astrocytes. 30 nM concentration of [^{3}H]-DA was added at different time stamps (60, 45, 30, 20, 15, 10, 5 min). Data from three separate experiments are represented as mean ± SEM (n = 9). The non-specific uptake was lower compared to the total uptake at each time stamp (t – test, p < 0.05).

3.2. Ouabain sensitivity of [^{3}H]-DA uptake into adult rat astrocytes

Cell cultures were preincubated at 37 °C with 0.1 and 1 mM concentration of ouabain for 20 minutes [45]. Ouabain is a cardiac glycoside that specifically targets and blocks the Na^+/K^+ ATPase, which is responsible for maintaining the electrochemical gradient of sodium and potassium ions across the cell membrane and is therefore crucial for transport mediated by uptake 1 transporters such as NET [59]. DA uptake was measured after 15 min incubation with 30 nM [^{3}H]-DA at 37 °C. DA uptake was significantly reduced in comparison to the control by 1 mM concentration of ouabain (51.811 ± 4.394 %), whereas 0.1 mM concentration of ouabain had no significant effect of DA uptake, which was reduced to 82.172 ± 8.631 % in comparison to the control 100 ± 8.019 % (Fig. 2).

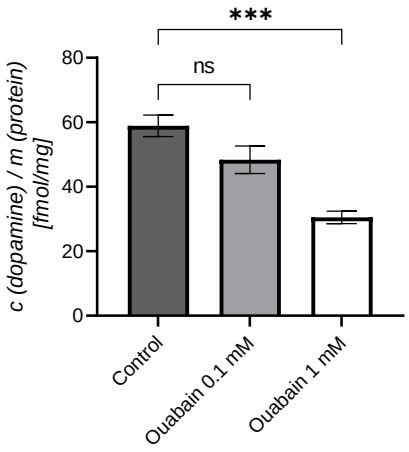


*Figure 2 Ouabain sensitivity of the total [^{3}H]-DA uptake into adult rat astrocytes. Astrocytes were incubated with 30 nM [^{3}H]-DA for 15 min at 37 °C under normal conditions and in the presence of 0.1 mM and 1 mM concentration of ouabain. Data are presented as mean of [^{3}H]-DA uptake ± SEM (n = 6), statistical significance was determined by Ordinary one-way ANOVA with Dunnett's correction, ns – non-significant, *** p = 0.0002.*

3.3. Concentration dependence of [^{3}H]-DA uptake in cultured adult rat astrocytes

Astrocytes were exposed to different concentrations of [^{3}H]-DA (0.03–750 µM) for a time span of 15 minutes, in line with the time course experiments, and the total (37 °C) and the non-specific (4 °C) DA uptake were measured. Specific uptake, from which the uptake kinetic characteristics were determined, was calculated as the difference between the total (37 °C) and non-specific (4 °C) uptake. DA uptake into cultured astrocytes, presented in Fig. 3 (a), was clearly dependent on DA concentration, however does not appear fully saturable at even the highest concentration of 750 µM. DA uptake velocity (Fig. 3, b) was calculated from the specific uptake and the time span of [^{3}H]-DA incubation (15 minutes). The kinetic parameters of uptake velocity were calculated using Michaelis – Menten equation. The apparent maximal uptake rate, V_{max}, of DA was calculated as 75.44 pmol/mg/min and the apparent Michaelis-Menten constant, K_m, was calculated as 1134 µM.

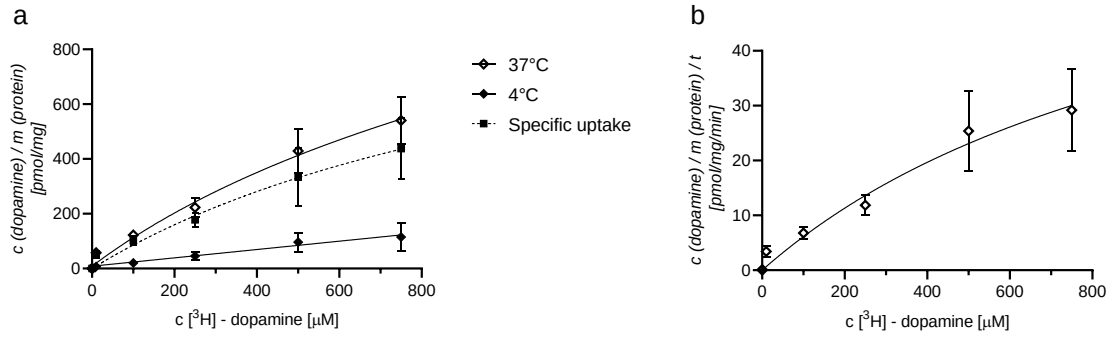


Figure 3 Concentration dependence of [^{3}H]-DA into cultured cortical adult rat astrocytes. DA uptake into astrocytes is concentration- and temperature-dependent. Experiments were repeated twice (n = 6), results are expressed as mean ± SEM. Specific uptake was calculated as the difference between total uptake (37°C) and non-specific uptake (4°C) (a). Uptake velocity was calculated from the Michaelis-Menten equation, apparent maximal uptake rate V_{max} = 75.44 pmol/mg/min and apparent Michaelis-Menten constant K_m = 1134 µM (b).

Astrocytes are capable of DA uptake, which is time-, temperature- and concentration- dependent. We presumed DA uptake is mediated by multiple transporters, therefore we examined the mRNA

expression of transporters involved in DA uptake, such as the main neuronal transporter of DA, DAT, NET and uptake 2 transporters OCT1, OCT2, OCT3 and PMAT.

3.4. mRNA expression of transporters involved in DA uptake in cortical adult rat astrocytes

We examined the mRNA expression of high-affinity and low-affinity transporters involved in DA uptake in adult rat astrocytes as well as adult rat cerebral cortex and kidney tissue serving as positive control. qPCR was performed after extraction of the total RNA from three weeks old confluent cultures and tissue samples, the latter were used as a positive control to validate our gene expression assays.

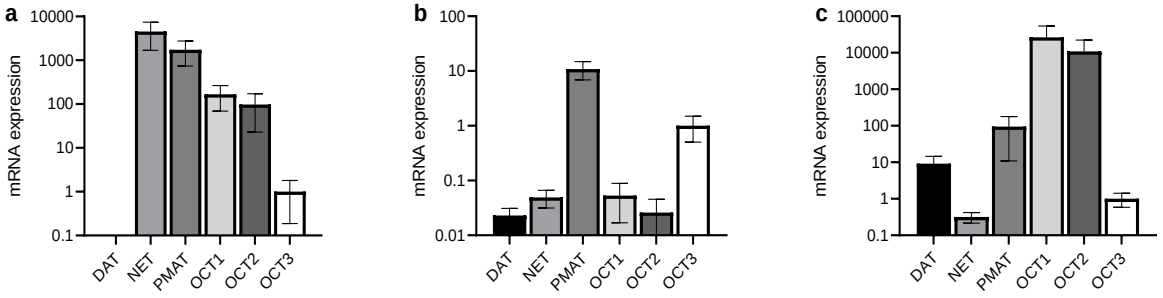


Figure 4 mRNA expression of transporters DAT, NET, PMAT, OCT1, OCT2, OCT3 in cortical adult rat astrocytes (a), cortical adult rat tissue (b) and adult rat kidney tissue (c). Results are expressed relative to the expression of OCT3. Data are represented as mean ± SEM (n = 6). mRNA expression is normalised to the mRNA expression of the endogenous control ß-actin.

Table 1 Pairwise comparison of transporter mRNA expression in adult rat cortical astrocytes. Statistical analysis was obtained using Brown-Forsythe ANOVA test with Dunnett's T3 multiple comparisons test.

Transporter comparison	*Mean Difference*	*Below threshold?*	*Summary*	*Adjusted P Value*
NET vs. PMAT	*0,0001253*	*No*	*ns*	*0,1315*
NET vs. OCT1	*0,0003156*	*Yes*	*	*0,0122*
NET vs. OCT2	*0,0003241*	*Yes*	*	*0,0118*
NET vs. OCT3	*0,0003357*	*Yes*	*	*0,0101*
PMAT vs. OCT1	*0,0001903*	*Yes*	***	*0,0002*
PMAT vs. OCT2	*0,0001988*	*Yes*	****	*<0,0001*
PMAT vs. OCT3	*0,0002105*	*Yes*	***	*0,0001*
OCT1 vs. OCT2	*8,52E-06*	*No*	*ns*	*0,9096*
OCT1 vs. OCT3	*2,02E-05*	*Yes*	**	*0,0036*
OCT2 vs. OCT3	*1,16E-05*	*No*	*ns*	*0,5895*

ns – nonsignificant

The mRNA expression profile of the transporters in adult rat cortical and kidney tissue (Fig. 4, b) was in line with our expectations. In the cortical tissue, among the low-affinity transporters, PMAT expression was most prominent, followed by OCT3 expression, which is among the three subtypes of OCTs considered the primary uptake 2 transporter in the CNS. In the kidney tissue, as expected, OCT1 and OCT2 mRNA expression exceeded OCT3 and PMAT mRNA expression. DAT and NET were present in both rat tissue samples, however NET expression was more prominent in rat cortical tissue than in kidney tissue. We observed a distinct mRNA expression profile in astrocyte cultures. Transporter expression was statistically significantly different (Brown-Forsythe ANOVA test: F* (DFn, DFd) = 117.0 (5.000, 4.785), $p < 0.0001$). The mRNA expression of all three OCTs in cortical adult rat astrocytes appears to be relatively low. Interestingly, among the three examined OCTs, OCT1 exhibits the highest mRNA expression, followed by OCT2 expression, while comparatively, OCT3 expression is nearly 100 times lower (Fig. 4, a). In astrocytes, mRNA expression of PMAT statistically significantly exceeded

mRNA expression of all three OCTs, however was lower than NET mRNA expression, contrarily to the rat cortical tissue, where PMAT mRNA expression was the most prominent. We have not been able to confirm DAT mRNA expression in cortical adult rat astrocytes, suggesting NET and PMAT might serve as the primary transporters involved in DA uptake into cultured cortical adult rat astrocytes. The statistical analysis of pairwise comparisons between mRNA expression of the examined transporters is displayed in Table 1.

3.5. The effect of inhibitor decynium-22 and antidepressants on [^{3}H] - dopamine uptake

After establishing that the main transporters that could transport DA into cultured rat astrocytes are NET and PMAT, we examined the effect of norepinephrine uptake inhibitors [60] nortriptyline and desipramine (Fig. 5, a), non-selective reuptake inhibitor amitriptyline (data not shown) as well as D22 (Fig. 5, b), a PMAT and OCT inhibitor [61,62]. Three weeks old cultures were preincubated with the transporter inhibitors for 20 minutes in the uptake buffer at 37 °C prior to the addition of 30 nM [^{3}H] –DA. The uptake was measured after 15 minutes.

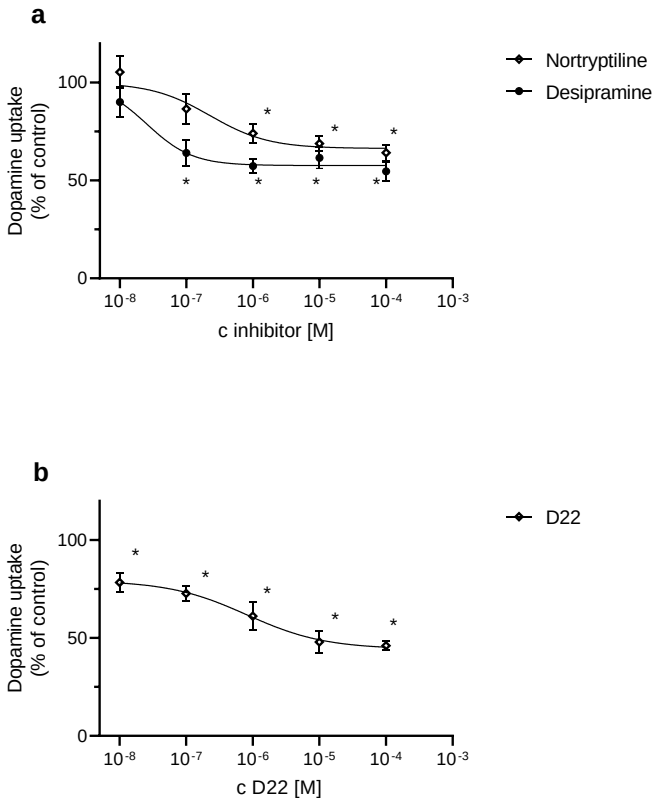


*Figure 5 Effect of antidepressants (a) nortriptyline and desipramine and D22 (b) on DA uptake into cultured adult rat astrocytes. Astrocytes were preincubated with these compounds for 20 minutes prior to the 15 min incubation with 30 nM concentration of [^{3}H]-DA at 37 °C (n = 9). Results are represented as percent (mean ± SEM) of the control. IC_{50} and pIC_{50} (calculated as the negative log of the corresponding IC_{50} values) of compounds inhibiting the total [^{3}H]-DA uptake into cultured adult rat astrocytes were calculated from the corresponding inhibition curves. Nortriptyline: IC_{50} = 0.225 ± 0.188 µM, pIC_{50} = 6.647, maximal inhibition: 64% in comparison to the control, desipramine: IC_{50} = 0.0254 ± 0.0140 µM, pIC_{50} = 7.595, maximal inhibition in comparison to the control: 54%, D22: IC_{50} = 0.813 ± 0.956 µM, pIC_{50} = 6.090, maximal inhibition in comparison to the control: 46%. Statistical significance was determined by Holm-Šidak method t-test vs control, * p < 0.05.*

Antidepressants desipramine and nortriptyline (0.01–100 µM) produced a significant inhibition of [^{3}H]-DA uptake from 0.1 µM onwards for desipramine and from 1 µM onwards for nortriptyline (Fig. 4), whereas amitriptyline had no significant effect on DA uptake. Nevertheless, these results suggest

NET may have a significant role in DA uptake into cultured astrocytes. D22 inhibited DA uptake at 0.01 µM already, uptake was reduced to approximately 80% (78.318 ± 5.153 %) in comparison to the control. The maximal total DA uptake inhibition by all three compounds reached only approximately 50%.

3.7 *Dopamine receptor expression in cortical adult rat astrocytes*

After establishing the mRNA transporter expression in our cell cultures of cortical adult rat astrocytes, we wanted to examine whether astrocytes express DA receptor mRNA. Results presented in Fig. 6 show a fairly low mRNA expression of both receptor subtypes (Welch's *t – test, p > 0.05*).

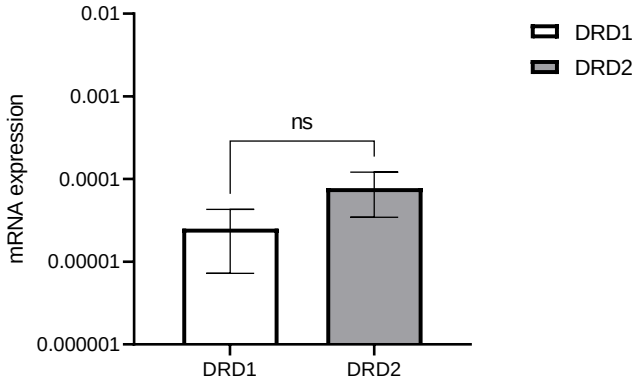


Figure 6 mRNA expression of DA receptors DRD1 and DRD2 in cortical adult rat astrocytes. Data are normalised to the endogenous control ß - actin and expressed as mean ± SEM (n = 10 for DRD1, n = 8 for DRD2, three separate experiments). Statistical significance of the difference between the expression of the two receptor subtypes was determined with Welch's t – test, p > 0.05, ns – non-significant.

The mRNA expression signal of DRD2 was at the limit of detection, therefore we proceeded to perform only qPCR analysis of DRD1 in further experiments.

3.7. Changes in mRNA expression of NET, PMAT and DRD1 in cortical adult rat astrocytes

In previous *in vitro* studies, concentrations of drugs such as (atypical) antipsychotics varied within the range of 1–100 µM [63]. Therefore, in this study we chose the concentrations of apomorphine, haloperidol and L-DOPA in this range, and based on preliminary experiments, decided on the largest concentration, that did not cause a reduction in cell viability.

Cortical adult rat astrocytes were treated with 100 µM concentration of apomorphine, haloperidol and L-DOPA for 24 hours. The viability of the cells at concentrations from 1 to 150 µM of the drugs was tested in a separate experiment using the MTS assay (Fig. 7). Astrocyte viability was significantly reduced (Two-way ANOVA, Bonferroni correction for multiple comparisons, ****$p < 0.0001$), by apomorphine at 150 µM concentration, whereas haloperidol and L-DOPA had no significant effect on astrocyte viability even at the highest concentration of 150 µM (Fig. 7).

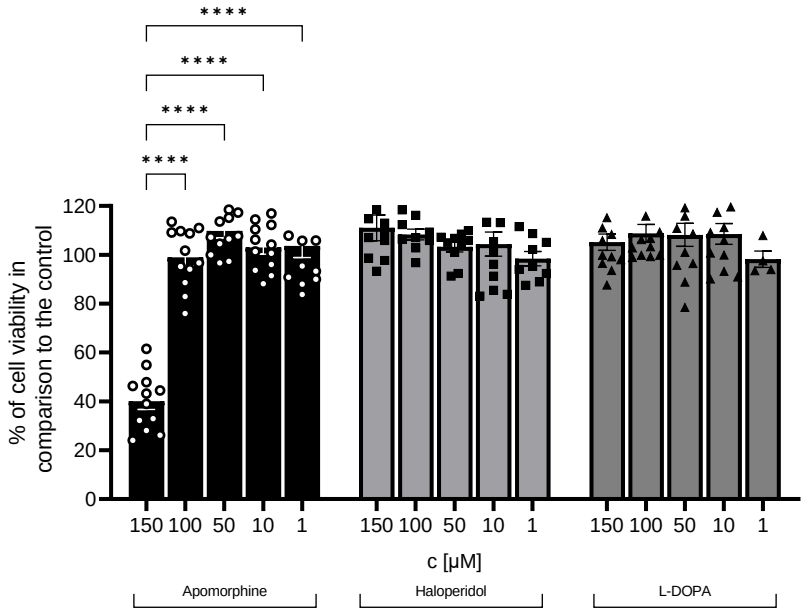


*Figure 7 Adult rat astrocyte viability after 24-hour treatment with apomorphine, haloperidol and L-DOPA was tested using the MTS assay and is presented as percent of the control ± SEM from three separate experiments (n = 12). Apomorphine induced a significant reduction in cell viability at the concentration of 150 µM; statistical significance was tested using Two-way ANOVA with Bonferroni correction for multiple comparisons, **** p = 0.0002.*

After establishing the effect the compounds on cell viability, we proceeded to test whether these drugs affect mRNA expression of transporters NET and PMAT, that have been shown to have the most prominent expression in our astrocyte cultures, as well as of receptor DRD1. We found a significant interaction between mRNA expression (NET ($F = 7.014$ (3.000, 12.19); $p = 0.0021$), PMAT ($F = 96.25$ (3.000, 7.238); $p < 0.0001$), DRD1 ($F = 10.07$ (3.000, 12.12); $p = 0.0013$)) and dopaminergic treatments (Fig. 8). This interaction was subsequently evaluated with pairwise comparison for each specific treatment.

Apomorphine induced significant upregulation of NET (3.452 ± 1.061 fold; t: 4.065, df: 20, $p = 0.0017$) PMAT (2.575 ± 0.225 fold; t: 8.705, df: 6.067, $p = 0.0004$) and DRD1 (4.733 ± 0.835 fold; t: 5.144, df:19, $p = 0.0105$), whereas the effect of L-DOPA and haloperidol on NET and DRD1 was statistically non-significant (Fig. 8). L-DOPA and haloperidol caused statistically significant downregulation in the expression of PMAT mRNA (L-DOPA: 0.604 ± 0.048 fold; t: 6.022, df: 8.145, $p = 0.0009$; haloperidol: 0.649 ± 0.058 fold; t: 4.872, df: 9.499, $p = 0.0025$) (Fig. 8).

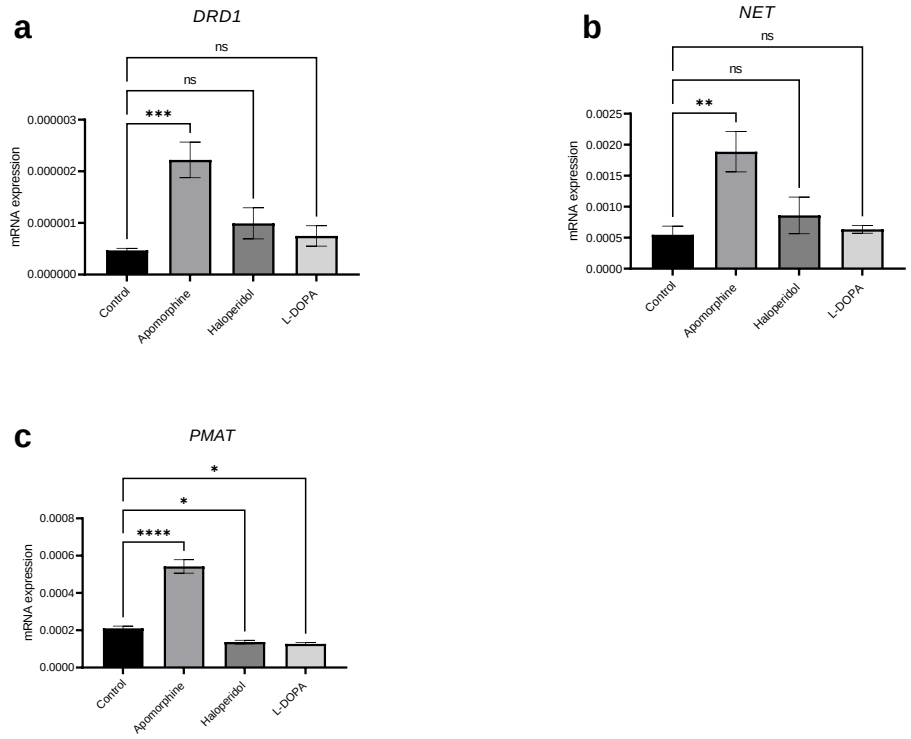


*Figure 8 Changes in mRNA expression of (a) DRD1, (b) NET and (c) PMAT after 24-hour treatment with apomorphine, haloperidol and L-DOPA. Data are expressed as mean ± SEM (n = 6) from two separate experiments and normalised to the mRNA expression of the endogenous control, ß – actin. Changes in mRNA expression expressed relative to the control: DRD1: control 1.000 ± 0.119, apomorphine 4.733 ± 0.835, haloperidol 2.115 ± 0.668, L-DOPA 1.596 ± 0.442; NET: control 1.000 ± 0.360, apomorphine 3.452 ± 1.061, haloperidol 1.573 ± 0.674, L-DOPA 1.161 ± 0.317; PMAT: control 1.000 ± 0.080, apomorphine 2.575 ± 0.225, haloperidol 0.649 ± 0.058, L-DOPA 0.604 ± 0.048. Statistical significance was determined by Brown-Forsythe ANOVA test with Dunnett's T3 multiple comparisons test: ns – nonsignificant, * $p < 0.05$, ** $p = 0.0025$, *** $p < 0.0005$.*

Discussion

We examined the pharmacological and molecular characteristics of DA uptake into cultured rat astrocytes of adult rats in the presence of MAO and COMT inhibitors, while earlier studies focused on glial cell cultures prepared from neonatal rats. Our results concerning pharmacological and molecular characteristics of DA uptake suggest that adult rat astrocytes possess a carrier system involving multiple transporters. This statement is supported by the fact that (i) astrocytes are capable of DA uptake, which is time-, temperature- and concentration-dependent, (ii) full inhibition of the Na^+/K^+-ATPase by ouabain caused a significant decrease (by roughly 50 %) of the DA uptake, indicating involvement of both Na^+-dependent and Na^+-independent uptake mechanisms, (iii) inhibition of DA uptake by antidepressants desipramine and nortriptyline as well as by D22, and lastly (iv) based on the mRNA expression of the examined transporters. The kinetic and qPCR results revealed the presence of a single low affinity-, high capacity uptake site, so there must exists very precise fine-tuned mechanism involving NET as well as high-capacity uptake carrier PMAT- mediated DA uptake in adult rat astrocytes. Additionally, we examined whether mRNA expression of transporters NET and PMAT as well as of receptor DRD1 is sensitive to treatment with dopaminergic drugs apomorphine, haloperidol and L-DOPA. After the cell treatments we observed changes in mRNA expression of both transporters, NET and PMAT, as well as of receptor DRD1 after treatment with apomorphine. Interestingly, haloperidol and L-DOPA had little effect on NET and DRD1 mRNA expression, albeit induced downregulation of PMAT mRNA expression.

DA transport into cortical astrocytes prepared from adult rats is a time-, concentration- and temperature-dependent process, which is consistent with multiple earlier experiments on neonatal rat astrocytes. Inazu et al. examined DA uptake into cortical astrocytes of neonatal rats and found DA uptake is time-, temperature- as well as concentration- dependent [45]. Uptake was not saturable, even at the highest concentration used (1 mM), whereas in the present study adult rat cortical astrocyte cultures exhibited characteristics of saturation, which was however not reached at the highest tested concentration of 750 µM. Pelton et al. work on primary astrocyte cultures from the cerebral hemispheres of neonatal rats also reached a similar conclusion, astrocytes took up DA in the concentration range of 0.1 to 100 µM, furthermore DA was metabolized in astrocyte by COMT and MAO. At 10^{-7} M, uptake of [^{3}H]-labelled DA was inhibited by the omission of Na^+, addition of ouabain or at low temperatures, whereas uptake at 100 µM was considerable but was Na^+-independent. Only Na^+-independent uptake was seen in primary cultures started from the meninges of neonatal rats [64]. Inazu et al. examined concentration-dependent inhibition of the Na^+/K^+-ATPase by ouabain and discovered DA uptake was reduced by roughly 50%. Uptake of 10^{-7} [^{3}H]-labelled DA in primary astrocyte cultures of neonatal rat brain performed by Semenoff et al. was 58% Na^+-dependent, indicating that astrocytes are capable of active uptake of DA [65,66]. Both results are in line with the results of our study, DA uptake was reduced to roughly 50% by 1 mM concentration of ouabain. Results of these studies indicate astrocytes possess both Na^+-dependent transport, crucial for DA uptake at lower concentrations of DA, and high-capacity, low-affinity Na^+-independent uptake carrier systems. Nevertheless, it is worth noting that some studies reported different results. Hansson et al. [67,68] and Hösli et al. [43] reported that astrocytes do not show saturable kinetics for DA, which they found to be Na^+-independent leading them to question the existenece of a high-affinity carrier system in astrocytes. Differences in Na^+-dependency of DA uptake can be explained by culture conditions and heterogeneity among astrocytes.

Supported by the data indicating astrocytes possess both Na^+-dependent and Na^+-independent carrier system for DA, we presumed the involvement of multiple uptake transporters, which was further

explored by qPCR. Although the main transporter mediating neuronal DA uptake is deemed to be DAT, our results in addition to the results of multiple other studies indicate that the main active transporter in rat astrocytes may be NET [38,45]. We have not been able to confirm the presence of DAT mRNA in our astrocyte cultures, on the other hand the mRNA expression of NET was the most prominent among the studied transporters and closely followed by PMAT, a high-capacity, low-affinity transporter. Due to comparatively low mRNA expression of the OCTs, which is in line with previous studies on both adult and neonatal cortical rat astrocyte cultures in our laboratory [34,69], we presume that the main transporter involved in active transport (uptake 1) of DA is NET and in non-specific transport (uptake 2) is PMAT. Inazu et al. reached a similar conclusion concerning the role of NET in cortical neonatal rat astrocyte in DA uptake as they were unable to confirm DAT mRNA expression, however they confirmed NET mRNA expression [38].

The results of our qPCR data are supported by inhibition experiments performed using tricyclic antidepressants nortriptyline (pK_i = 8.2 for human NET [70,71]) and desipramine (pK_i = 8.133 in COS-7 cells expressing rat NET [39]), both NET selective inhibitors as well as an OCT and PMAT inhibitor D22 (pK_i in MDCK cells stably expressing PMAT was determined as 7.0 [62,72]). In the present study, the pIC_{50} of desipramine was calculated at 7.595 and at 6.647 for nortriptyline. The calculated pIC_{50} of D22 was determined to be around 6.090. Selective inhibitors for the high-capacity, low-affinity DA transporters, such as the OCTs and PMAT, are lacking, however due to the low mRNA expression of the OCTs, we may assume that their role in astrocyte DA uptake is negligible in comparison to the role of PMAT. The importance of NET in astrocyte DA uptake was confirmed also by Takeda et al. who determined Na^+-dependent DA uptake was significantly inhibited by a NET selective inhibitor nisoxetine (100 nM concentration), whereas GBR1235, a potent DAT inhibitor, inhibited DA uptake at 10 µM in addition inhibited DA uptake in Na^+-free conditions, whereas nisoxetine did not, indicating the importance of NET in astrocyte active DA transport [44].

Multiple studies have reported mRNA expression of both DRD subtypes in astrocytes [51,73], however reports of DRD expression vary between animal species and the studied brain region. Perhaps astrocytes in different brain regions may express different DA receptors, or different DA receptors serve distinct brain-area-specific signalling [74]. Our results indicate a fairly low expression of both receptor subtypes, however mRNA expression of DRD2 was at the limit of detection of our qPCR analysis method. Activation or blockade of brain DA receptors has been embraced as a mechanism for the therapeutic efficacy of dopaminergic drugs. Although this concept is correct in principle it may be too simplistic. Receptors are functionally linked with intracellular molecular networks that control membrane excitability, as well as neurotransmitter synthesis, release, and metabolism, and by these mechanisms, neuronal cells can regulate their own activity. Thus, drugs targeting DRDs can impact neurotransmitter synthesis, release, and metabolism [75]. Astrocyte DRDs have been shown to induce fluctuations in intracellular signalling molecules in different brain areas. Some studies have connected these changes to activation of DRD1 [76], some to DRD2 [31,77], by various dopaminergic compounds. These changes have yet to be thoroughly explored, not only how astrocyte DA receptors respond to different stimuli, but also whether they lead to any possible changes in astrocyte DA transport. Therefore, we decided to examine whether treatment of our adult rat astrocyte cultures with high concentrations of apomorphine, haloperidol and L-DOPA might induce changes in the expression of DRD1 as well as transporters involved in astrocyte DA uptake, NET and PMAT.

We used three different compounds: apomorphine, haloperidol and L-DOPA. Apomorphine, a potent DRD1/DRD2 agonist, is currently used as an antiparkinsonian drug [78]. L-DOPA is a prodrug of DA that is administered to patients with Parkinson's due to its ability to cross the blood-brain barrier. Once

past the blood-brain barrier, L-DOPA is metabolized to dopamine and supplements the low endogenous levels of dopamine to treat symptoms of Parkinson's. L-DOPA can interact with DRD1 or DRD2 receptors independent of its conversion to endogenous dopamine [79]. Haloperidol competitively blocks post-synaptic DRD2 in the brain, eliminating DA neurotransmission and leading to the relief of delusions and hallucinations that are commonly associated with psychosis. It acts primarily on the DRD2 and has some effect on DRD1 [80].

Apomorphine induced changes of all examined samples (Fig. 8), the greatest change was observed in DRD1, followed by two-fold upregulation of NET and PMAT. Haloperidol has been found to change the expression of neuronal D1 and D2 receptors [80-82], however interestingly, in our study, it had no effect on astrocyte DRD1 receptor expression. L-DOPA has been found to upregulate DRD1 sensitivity and promote DA release through the reversal of DAT [79], however it did not affect its expression in astrocytes. Although haloperidol and L-DOPA did not induce any significant change in the expression of NET mRNA, we observed a significant downregulation of PMAT mRNA expression. Haloperidol has been found to inhibit hPMAT at micromolar concentrations [83], whereas we have not found similar data on L-DOPA in regard to PMAT. Interestingly, all three compounds affected the expression of the high-capacity transporter PMAT, and only apomorphine induced upregulation of both NET and DRD1.

Astrocytes are involved in regulation of DA homeostasis in the tripartite dopaminergic synapse and respond to various dopaminergic compounds through changes in gene expression of transporters involved in DA uptake and DRDs. The exact mechanism behind these changes is in need of further research.

Conclusion

Astrocytes contribute to DA homeostasis and are capable of DA clearance and metabolism. DA uptake into astrocytes is not mediated by the uptake 1 transporter DAT, but by NET and high-capacity, low-affinity transporter PMAT. Astrocytes are sensitive to treatment with dopaminergic drugs, the impact of the changes in mRNA expression in astrocytes as well as in the DA homeostasis need further research.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

VS and MK designed the project, VS and KD performed and analysed the measurements, VS and MK interpreted the data, wrote and edited the manuscript.

Data availability statement:

Experimental data are available from the first or corresponding author upon reasonable request.

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