

The mammalian purine salvage pathway as an exploitable route for cerebral bioenergetic support after brain injury

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ABSTRACT

Purine-based molecules play ancient, fundamental, and evolutionarily-conserved roles across life on Earth, ranging from DNA and RNA, to the universal energy currency, ATP. In mammals, the two primary routes for the synthesis of the adenine nucleotides ATP, ADP and AMP, and, as a consequence, the major bioactive metabolite adenosine, are the de novo purine biosynthesis (DNPB) pathway, and the purine salvage pathway (PSP). Of the two, the PSP dominates in both the mammalian brain and heart. This is because the PSP utilizes the breakdown products of ATP, occasioned by the high energy demands of these organs, to rapidly regenerate adenine nucleotides. This resynthesis route, while efficient and energetically favourable, leaves these organs vulnerable to loss of salvageable metabolites, with the potential for protracted depletion of the means to synthesize ATP, and the ability to deploy neuro- and cardioprotective adenosine. Having previously shown that hippocampal cellular ATP and adenosine release can be increased by supplying substrates for the PSP (D-ribose and adenine), we now explore the expression of DNPB and PSP enzymes in hippocampal neurons and astrocytes based on available transcriptomic data. We find that key enzymes of the PSP are expressed at higher levels than those in the DNPB pathway, and that PSP enzymes are expressed at higher levels in neurons than in astrocytes. These data reflect the importance of the PSP in the mammalian brain and imply that pharmacological targeting of the PSP may be particularly beneficial to neurons at times of metabolic stress.

This article is part of the Special Issue on 'Purinergic Signaling: 50 years'.

1. Introduction

Purines are among the most abundant metabolites in mammalian cells. They serve as nucleic acid building blocks for DNA and RNA, can be found in cofactors (NADH and coenzyme A), which promote cell proliferation and survival, are involved in intracellular (cAMP, cGMP) and extracellular signaling (ATP, ADP, adenosine, inosine), and provide cells with the necessary energy to function in the form of ATP (Jinnah et al., 2013; Pedley and Benkovic, 2017). This widespread role of purine-based molecules likely reflects their ancient origin. Meteorites have been shown to contain a wide variety of purines including adenine, hypoxanthine and xanthine (Hayatsu, 1964; Callahan et al., 2011), and indeed ribose, the sugar backbone of DNA, RNA and ATP (Furukawa et al., 2019). More recently, the discovery of pyrimidines in meteorites (Oba et al., 2022) completes the set of nucleobases required for the base pairs in DNA, suggesting that life on Earth may have been seeded from extra-terrestrial sources during the Earth's early development.

Given the importance of purines in cellular metabolism and activity,

two specialised pathways, which don't depend upon extra-terrestrial intervention, have developed to provide these molecules: the de novo purine biosynthesis (DNPB) pathway and the purine salvage pathway (PSP; Fig. 1). The DNPB pathway, in which the purine moiety is sequentially constructed upon a ribose phosphate derived from phosphoribosyl pyrophosphate (PRPP) to initially yield inosine monophosphate (IMP), utilizes six enzymes, ten intermediary steps, substrates from other biochemical pathways, and ATP (Pareek et al., 2021) (Fig. 1). Two further steps, requiring GTP and aspartate, are necessary for the conversion of IMP into an adenine nucleotide, AMP. Mitigation of these multi-step and multi-substrate demands may arise, including in neurons (Williamson et al., 2017; Mangold et al., 2018), from the assembly of DNPB components (enzymes, metabolites and substrates) into a complex known as the purinosome that favours kinetic reactions through the concentration of enzymes and substrates in close proximity to mitochondria (Pareek et al., 2020). As such, the DNPB is highly energy intensive, does not increase its output quickly after metabolic stress, and is minimally operational in the mature mammalian central nervous

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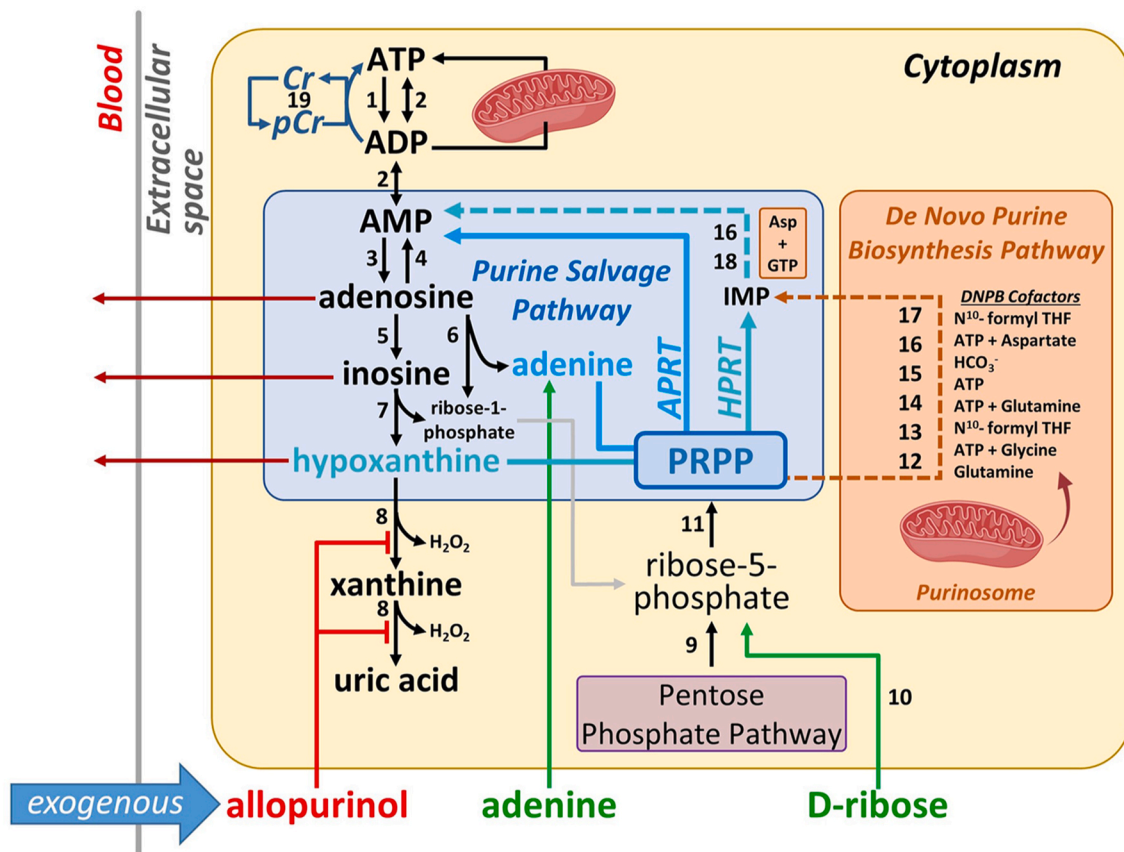


Fig. 1. Purine metabolism and the purine salvage and de novo purine biosynthesis pathways of mammalian cells. The purine salvage pathway (PSP) recycles ATP metabolites for resynthesis of ATP, initially in the form of AMP from which ADP can be converted, in the cytosol or via mitochondria, to ATP. The PSP also limits the loss of ATP metabolites from both the cell, and potentially into the blood stream, and from conversion into non-salvageable metabolites. The alternative de novo purine biosynthesis (DNPB) pathway utilizes a multi-enzyme, multi-cofactor process involving mitochondria arranged in a molecular complex known as the purinosome. The cofactors are utilized in reverse order as the synthesis of IMP progresses. Exogenous compounds can be administered to either increase substrate availability (green arrows; adenine and D-ribose), or inhibit key enzymes (red lines; allopurinol) 1, ATPases; 2, adenylate kinase; 3, 5'nucleotidase; 4, adenosine kinase; 5, adenosine deaminase; 6, purine nucleoside phosphorylase LACCI/Famin; 7, purine nucleoside phosphorylase; 8, xanthine oxidase; 9, ribose 5-phosphate isomerase; 10, ribokinase; 11, phosphoribosyl pyrophosphate synthetase; 12, phosphoribosyl pyrophosphate amidotransferase; 13, trifunctional purine biosynthetic protein adenosine-3; 14, phosphoribosylformylglycinamide synthase; 15, phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase; 16, adenylosuccinate lyase; 17, bifunctional purine biosynthesis protein ATIC; 18, adenylosuccinate synthetase; 19, creatine kinase. The conversion of ribose-1-phosphate to ribose-5-phosphate (grey arrow) is by phosphopentomutase. APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; PRPP, phosphoribosyl pyrophosphate; N¹⁰-formyl THF, N¹⁰-formyltetrahydrofolate; Asp, aspartate; GTP, guanosine triphosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

system (Gerlach et al., 1971; Allsop and Watts, 1980; Barsotti et al., 2002; Pareek et al., 2021).

In contrast, the PSP (Murray, 1971) (Fig. 1) recycles the products of ATP metabolism - adenine, hypoxanthine and ribose-1-phosphate - into AMP, either directly, or following the conversion of IMP generated via the PSP or the DNPB pathway. As such, the PSP, compared to the DNPB pathway is rapid, energetically frugal and can swiftly increase in activity in stress situations when substrate availability is increased (Ipata et al., 2011; Ipata and Balestri, 2013; Nyhan, 2014). The PSP thus capitalizes on the products of ATP degradation and is mainly catalysed through two enzymes: adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). APRT can directly convert adenine to AMP, and HPRT can directly convert either hypoxanthine to IMP or guanine to GMP, the former of which can then be catalysed to AMP through two further enzymatic steps requiring two substrates (Fig. 1). Both APRT and HPRT reactions depend upon PRPP to supply the necessary ribose-5-phosphate to adenine and hypoxanthine, respectively. PRPP arises via the action of phosphoribosyl pyrophosphate synthetase 1 & 2 (PRPS1 & 2) on ribose-5-phosphate. Ribose-5-phosphate is supplied through the pentose phosphate pathway, the isomerization of ribose-1-phosphate, which can be derived from

adenosine or inosine, or directly through the phosphorylation of D-ribose via the action of ribokinase.

The importance of the PSP cannot be understated: not only is it the primary route for adenine nucleotide synthesis in the mammalian brain, the enzymes concerned are cytosolic and extramitochondrial, which indicates that the PSP may operate under conditions of mitochondrial dysfunction caused by cellular stress, such as cerebral ischemia. Under these conditions, the fact that certain isoforms of adenylate kinase (which converts AMP to ADP and ATP) are also cytosolic and found in brain (Panayiotou et al., 2014), suggests that some non-mitochondrial provision of cellular ATP could be made under stress conditions. Moreover, such a non-mitochondrial ATP generating system may be of value in highly morphologically polarised neurons where sites of energy-demanding activity (eg dendritic spines) may be supplied locally through glycolysis and PSP- and adenylate kinase-generated ATP. This is especially the case given that mitochondria are only found in a small percentage of cortical and hippocampal dendritic spines (Chicurel and Harris, 1992; Kasthuri et al., 2015; Frenguelli, 2019) and in fewer than 50% of presynaptic terminals (Shepherd and Harris, 1998; Smith et al., 2016).

The PSP performs other important tasks in addition to recycling ATP

Table 1List of genes used to visualize DNPB and the PSP with their matching EC protein codes. Data collected from [UniProt.org](https://www.ebi.ac.uk/UniProt/) and [PubChem](https://pubchem.ncbi.nlm.nih.gov/).

Gene Name	Protein	EC N°	Role
9030625A04Rik	Purine nucleoside phosphorylase LACC1/Famin	2.4.2.1 2.4.2.28 3.5.4.4	PSP
Adsl	Adenylosuccinate lyase	4.3.2.2	DNPB/PSP
Adss	Adenylosuccinate synthetase	6.3.4.4	DNPB/PSP
Ak1	Adenylate kinase isoenzyme 1	2.7.4.3 2.7.4.6	Adenine nucleotide synthesis
Ak5	Adenylate kinase isoenzyme 5	2.7.4.3 2.7.4.6	Adenine nucleotide metabolism
Ampd1	AMP deaminase 1	3.5.4.6	Adenine nucleotide metabolism
Ampd2	AMP deaminase 2	3.5.4.6	
Ampd3	AMP deaminase 3	3.5.4.6	
APRT	Adenine phosphoribosyltransferase	2.4.2.7	PSP
Atic	Bifunctional purine biosynthesis protein ATIC	2.1.2.3 3.5.4.10	DNPB
Atp1a1	Sodium/potassium-transporting ATPase subunit alpha-1	7.2.2.13	Adenine nucleotide metabolism
Atp1a2	Sodium/potassium-transporting ATPase subunit alpha-2	7.2.2.13	
Atp1a3	Sodium/potassium-transporting ATPase subunit alpha-3	7.2.2.13	
Ckb	Creatine kinase B-type	2.7.3.2	Adenine nucleotide synthesis
Gart	Trifunctional purine biosynthetic protein adenosine-3	2.1.2.2 6.3.3.1 6.3.4.13	DNPB
Hprt	Hypoxanthine-guanine phosphoribosyltransferase	2.4.2.8	PSP
Nt5c1a	Cytosolic purine 5'-nucleotidase (cN-IA; AMP-preferring)	3.1.3.5	Adenine nucleotide metabolism
Nt5c2	Cytosolic purine 5'-nucleotidase (cN-II; IMP-preferring)	3.1.3.5	Adenine nucleotide metabolism
Paics	phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase	4.1.1.21 6.3.2.6	DNPB
Pfas	Phosphoribosylformylglycinamide synthase	6.3.5.3	DNPB
Pgm2	phosphopentomutase	5.4.2.7	DNPB/PSP
Pnp	Purine nucleoside phosphorylase	2.4.2.1	PSP
Ppat	Phosphoribosyl pyrophosphate amidotransferase	2.4.2.14	DNPB
Prps1	Ribose-phosphate pyrophosphokinase 1	2.7.6.1	DNPB/PSP
Prps2	Ribose-phosphate pyrophosphokinase 2	2.7.6.1	DNPB/PSP
Rbks	Ribokinase	2.7.1.15	DNPB/PSP
Rpia	Ribose-5-phosphate isomerase	5.3.1.6	DNPB/PSP
Slc29a1	Equilibrative nucleoside transporter 1	Adenine/hypoxanthine uptake	
Slc29a2	Equilibrative nucleoside transporter 2	Adenine/hypoxanthine uptake	
Xo	Xanthine dehydrogenase/oxidase	1.17.1.4 1.17.3.2	Purine metabolism

metabolites into AMP and hence ATP. It does so in two ways: in preventing adenosine, inosine, and hypoxanthine from entering the bloodstream where they would be lost from salvage, and sequestering hypoxanthine before its xanthine oxidase-mediated conversion to the unsalvageable xanthine and uric acid. There is evidence, however, that both these scenarios are breached during cerebral ischemia: the activity of cytosolic 5' nucleotidase (cN-IA), which converts AMP to adenosine, is greatly increased during ATP-depleting conditions (Ipata and Balestri, 2013), likely contributing to the extracellular elevation of purines (Dale and Frenguelli, 2009), their appearance in peripheral blood (Fisher et al., 2019; Frenguelli and Dale, 2020), and the accumulation of cerebral xanthine and uric acid observed during these events with mass spectrometry imaging techniques (Liu et al., 2014; Mulder et al., 2016).

Given this dual loss of salvageable metabolites (to blood stream and xanthine and uric acid), we previously sought to restore the pool of PSP substrates by delivering D-ribose and adenine ("RibAde") to brain slices, which experience ATP-depleting ischemia and trauma in their preparation (Frenguelli, 2019). In keeping with studies in the heart, with its high energetic demands, reliance on the PSP and ATP-depleting cardiac insults (Zimmer, 1998; Frenguelli, 2019), RibAde elevated brain slice ATP levels to the point that when the cut dead edges of slices were taken into account, ATP values reached those reported previously in vivo (zur Nedden et al., 2011). This elevated cellular pool of ATP resulted in greater release and inhibitory actions of adenosine under physiological stimulation (zur Nedden et al., 2011), and in vitro models of cerebral ischemia (zur Nedden et al., 2014) and seizure activity (Hall and Frenguelli, 2018). Moreover, when administered in vivo, RibAde or RibAde plus the xanthine oxidase inhibitor, allopurinol (RibAdeAll) showed

encouraging evidence of reducing brain damage and improving recovery in an animal model of stroke (Faller et al., 2017).

However, the cellular site of action of RibAde or RibAdeAll in these experimental observations is not clear, and could potentially involve both neurons and astrocytes, based on the extent to which they express enzymes for the PSP, and indeed DNPB pathway. The aim of this study, therefore, was to assess the expression of PSP and DNPB pathway enzymes in both neurons and astrocytes, with a view to offering new insights into purine synthesis and metabolism in two important cell types in the brain, and to better understand where the ATP-restoring properties of RibAde and RibAdeAll may have their influence.

Based on published transcriptomic data (Zhang et al., 2014), we show that key enzymes of the PSP are expressed at a higher level than those in the DNPB pathway, and that neurons have higher levels of key PSP enzymes than astrocytes. Notably, while neurons have slightly higher levels of APRT compared to astrocytes (~1.6-fold), there is an appreciable 5.6-fold greater expression of HPRT in neurons, as well as in two cytosolic adenylate kinase isoforms (AK1, ~3.5-fold and AK5, ~39-fold) that convert AMP to ADP and ATP. These data confirm the importance of the PSP in the mammalian brain, and in particular in neurons, and provide a rational basis for the targeting of the PSP in acute and chronic conditions affecting brain energy metabolism, such as cardiac arrest, stroke, traumatic brain injury, convulsive and non-convulsive status epilepticus, and neurodegenerative disorders.

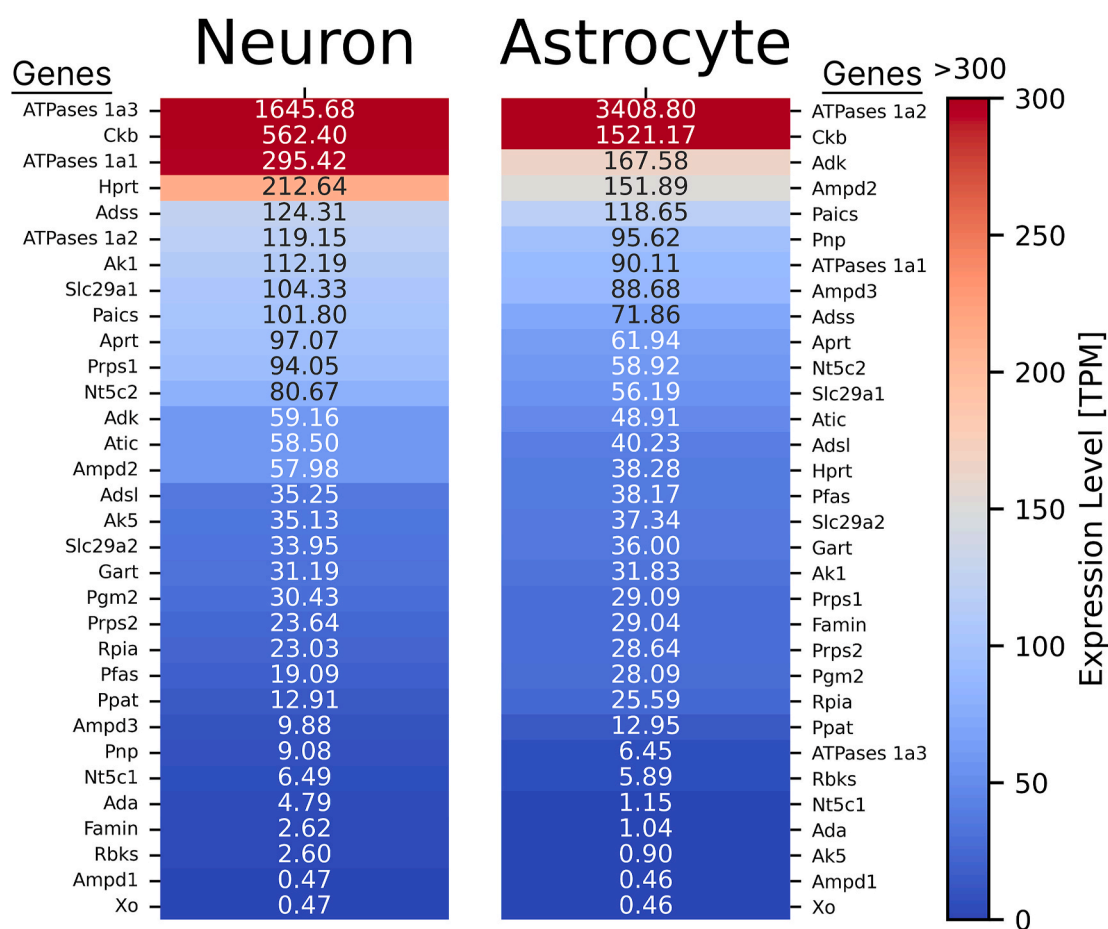


Fig. 2. Comparison of gene expression of key components in the synthesis or metabolism of purines. Left hand panel shows gene expression profile in neurons, and right panel shows that in astrocytes. Note similarities in high expression of ATPase subunits and creatine kinase (Ckb) in both, and the very low levels of xanthine oxidase (Xo). Values for each gene are given in TPM and colour-coded as per the scale on the right, with TPM ≥ 300 shown in dark red. Due to a change in enzyme nomenclature for consistency between murine and human data, the Pgm2 values correspond to the corresponding mouse Pgm1 in the RNA-Seq dataset obtained prior to the change in nomenclature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Methods

2.1. RNA expression data

Zhang and colleagues (Zhang et al., 2014) generated, and made publicly available, a RNA-Seq transcriptome database (GSE52564) of purified cell types from the cerebral cortices of mice, which included astrocytes and neurons from litters of postnatal day 7 (P7) mice. Our analysis of purine synthesis and metabolising enzymes is based upon this database.

2.2. Data normalization

For this work transcripts per million base pairs (TPM) was chosen as unit for normalizing raw expression data across samples. The TPM values were calculated as follows (1)

$$TPM = \frac{\text{Counts}}{\text{length of gene}} \times \left(\frac{1}{\text{Sum of all rates}} \right) \times 10^6 \quad (1)$$

Counts/length of genes results in the rate for a single gene, which is then multiplied by the sum of all these rates, and the result is again multiplied by a million to achieve easily workable numbers (Li et al., 2010).

Values already normalized as FPKM were converted to TPM through the following calculation (2) (Pachter, 2011).

$$TPM = \left(\frac{FPKM}{\text{sum FPKM}} \right) \times 10^6 \quad (2)$$

2.3. Genes

The genes selected for comparison (Table 1) are known genes contributing to purine synthesis and metabolism and include the key elements of the PSP and DNPB pathway. Additionally, the genes for the catalytic subunits of the Na^+/K^+ ATPase were chosen as a proxy for energy usage of the cell. The transporter for adenine and related nucleosides was also included to better understand transport of PSP substrates.

2.4. Analysis

For this work Python was used with multiple libraries to better process and visualize data. Numpy, Pandas, and Pylab were used to dissect and analyse the data. Seaborn and Matplotlib were used to help in the visualization of the analysed data, while Cytoscape was used to create diagrams of enzymatic pathways in which the colour of lines reflected gene expression levels. To allow a wide dynamic colour range for data presentation, and for ease of comparison across data sets (heat maps and pathways) a threshold of 300 TPM (Figs. 2–6) or 40-fold differences (Fig. 7) was set. Values above this maximum appeared deep red.

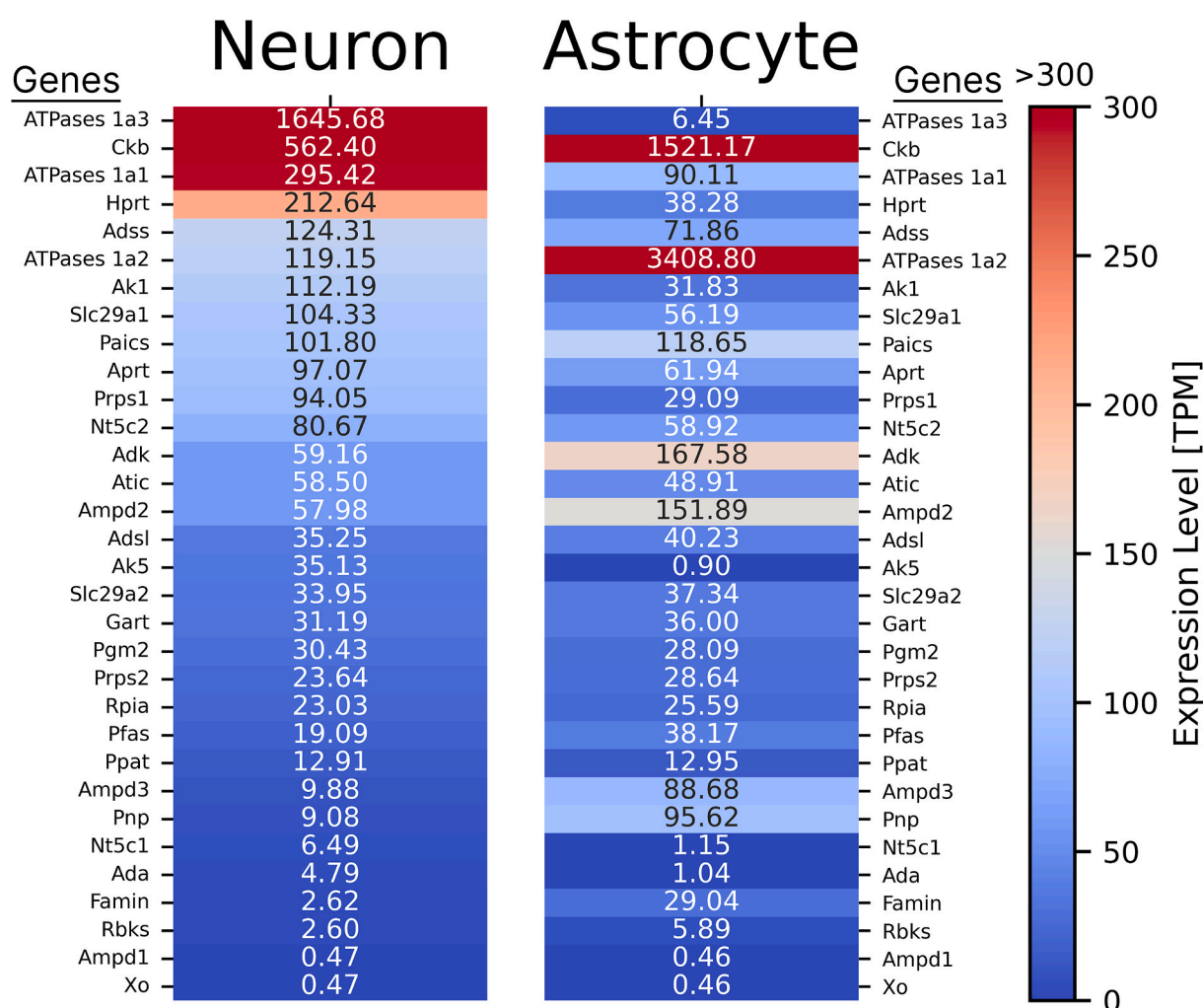


Fig. 3. Side by side comparison of expression levels of genes expressed in purine synthesis and metabolism by neurons and astrocytes, ordered with respect to the highest expression levels in neurons. Neurons have appreciably higher levels of HPRT and the adenylate kinases (AK1 and AK5). Maximum threshold for colour scaling was set at 300 TPM.

2.5. Custom scripts

Custom scripts were especially developed to automatically sift through the databases and extract the desired data. They are designed to work with similar datasets and thus can enable further metabolic analysis and visualization through Cytoscape. Scripts are available on Github under: <https://github.com/Phocasola/Metabolic-Pathway-Scripts>.

3. Results

3.1. Comparison of purine synthesis and metabolising enzymes in neurons and astrocytes

Our bioinformatic analysis of purine synthetic and metabolising enzymes, based on a previously published RNA-Seq database (Zhang et al., 2014), revealed a wide range of expression for the genes of interest (Table 1; Fig. 2; 4 orders of magnitude). Importantly, and based on the analysis by Zhang and colleagues, who set a statistical threshold at FPKM values of 0.1 (TPM of 0.45), these genes were all expressed at statistically-significant levels (>99% confidence).

Our analysis, which sought to have a proxy for ATP consumption, as well as ATP production, included the catalytic α subunits of Na^+/K^+ ATPases as exemplar major consumers of ATP, especially in neurons (Lezmy et al., 2021). Accordingly, Na^+/K^+ ATPase subunits featured prominently amongst the most highly-expressed of the genes under

investigation in both neurons and astrocytes (Fig. 2). Significantly, the $\alpha 3$ subunit, regarded as being predominately neuronal (Shrivastava et al., 2020), was expressed at levels ~ 250 -fold higher than those in astrocytes, with the reverse expression (~ 30 -fold higher) for the astrocyte-specific $\alpha 2$ subunit in astrocytes (Shrivastava et al., 2020). The ~ 3 fold greater expression of Adk in astrocytes compared to neurons is consistent with immunohistochemical location of this enzyme (Studer et al., 2006; Etherington et al., 2009). This differential expression confirms the nature of the two cell types under investigation. Also highly expressed in both neurons and astrocytes was the cytosolic b (brain) isoform of creatine kinase (Hanna-El-Daher and Braissant, 2016), which was expressed at 3-fold higher levels in astrocytes compared to neurons. This suggests that the buffering capacity of creatine kinase in maintaining cellular ATP levels is important to both cell types.

Of the enzymes responsible for introducing the ribose sugar moiety into the PNP and DNPB pathway, Rpia and Rbks, and Prps1 and Prps2, Rbks (ribose \rightarrow ribose-5-phosphate) was expressed 2.3 fold higher in astrocytes than neurons, whereas Prps1 (ribose-5-phosphate \rightarrow PRPP) was found at 3.2-fold higher levels in neurons. Similar levels of expression were found for Pgm2, which converts adenosine and inosine-derived ribose-1-phosphate into ribose-5-phosphate for conversion by Prsp.

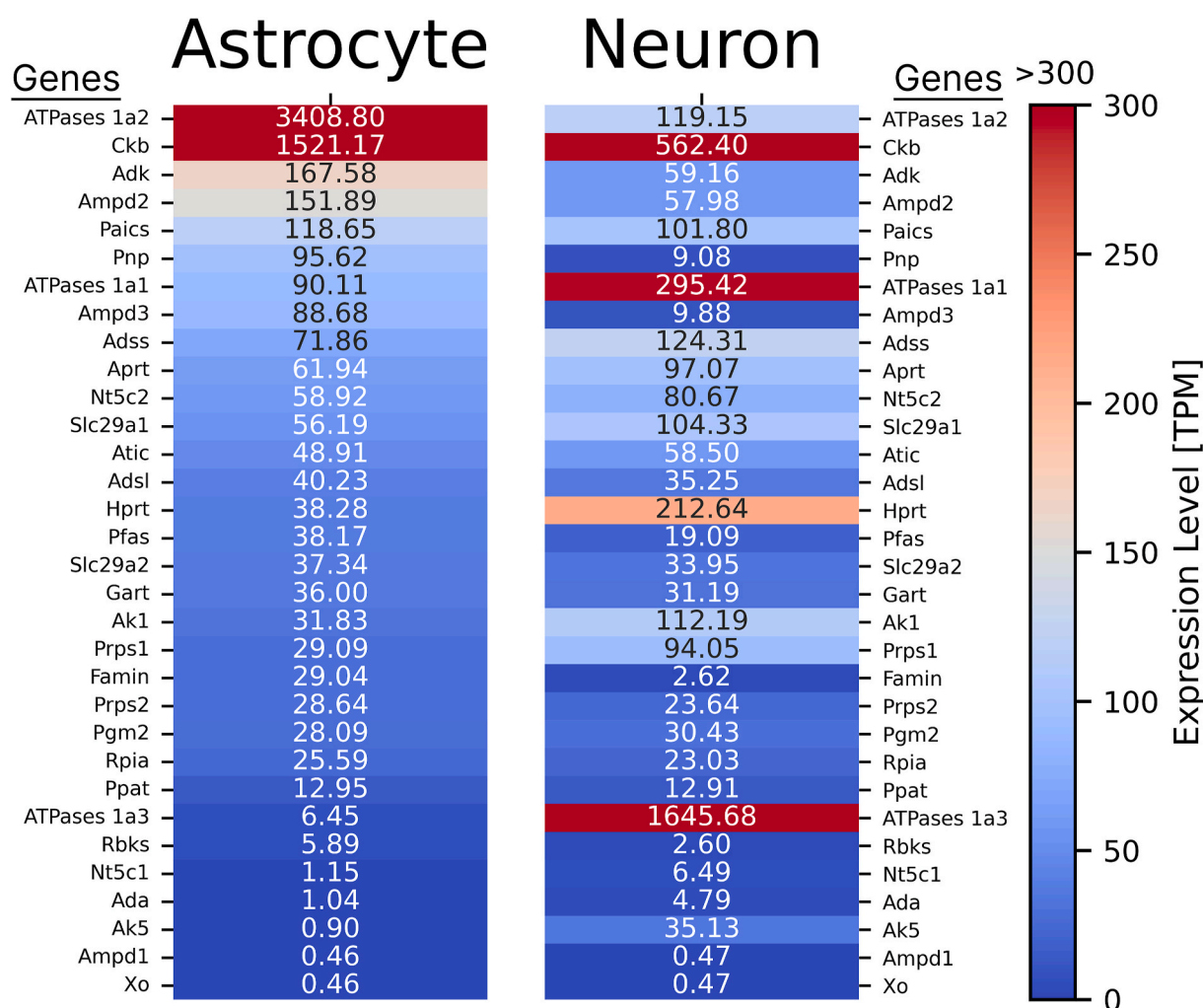


Fig. 4. Side by side comparison of expression levels of genes expressed in purine synthesis and metabolism by neurons and astrocytes, ordered with respect to the highest expression levels in astrocytes. Maximum threshold for colour scaling was set at 300 TPM.

3.2. Comparable expression of DNPB pathway enzymes and xanthine oxidase between neurons and astrocytes

Apart from Pfas (2-fold increase in astrocytes), there was little difference in the expression of the six core enzymes of the DNPB pathway between neurons and astrocytes (Fig. 2), but a slight enrichment (1.7 fold) for Adss in neurons, one of the two enzymes (along with Adsl) that convert IMP to AMP. Interestingly, xanthine oxidase was expressed at the lowest level in both neurons and astrocytes and at the threshold of statistically-significant expression (0.1 FPKM; 0.45 TPM) defined by Zhang and colleagues (Zhang et al., 2014).

3.3. Differential expression of PSP pathway enzymes between neurons and astrocytes

Fig. 3 shows the ranking of astrocyte enzymes relative to their expression levels in neurons, while Fig. 4 shows the ranking of neuronal gene expression relative to that in astrocytes. Of the enzymes specifically related to the PSP, HPRT was expressed at levels ~5.5-fold greater in neurons than astrocytes, which is consistent with earlier observations (Jinnah et al., 1992), while there was a modest elevation in neurons of APRT compared to astrocytes (~1.6-fold). Thus, neurons may have a preferential reliance on the PSP to synthesize ATP. This is further reinforced by the greater expression (3.2-fold) of Prps1 in neurons, which converts ribose-5-phosphate to PRPP, and of cytosolic isoforms of

adenylate kinase, which interconvert AMP, ADP and ATP (Panayiotou et al., 2014), in neurons compared to astrocytes (by ~3.5 and ~40 fold for Ak1 and Ak5, respectively). While both cell types possess plasma membrane uptake transporters for adenine and hypoxanthine (Slc29a1/ENT1 and Slc29a2/ENT2) (Yao et al., 2011; Young et al., 2013), ENT1 is expressed almost 2-fold higher in neurons than astrocytes.

Astrocytes (Fig. 4) predominately expressed enzymes related to the metabolism of purines, notably AMP deaminase (3.5 fold increase), purine nucleoside phosphorylase (~10.5 fold increase) and FAMIN (~11 fold increase). Of these, FAMIN is particularly interesting, as it is a recently described multifunctional enzyme with adenosine deaminase, purine nucleoside phosphorylase and S-methyl-5'-thioadenosine phosphorylase and adenosine phosphorylase activity (Cader et al., 2020). FAMIN is thus capable of liberating adenine from both S-methyl-5'-thioadenosine, the previously understood source of adenine in eukaryotic cells, and adenosine itself.

The gene expression counts have been mapped onto the PSP and DNPB pathway for neurons astrocytes in Figs. 5 and 6, respectively, with the differential expression (fold-differences; neurons > astrocytes and astrocytes > neurons) shown in Fig. 7A and B, respectively. Fig. 7A shows the emphasis on ATP synthesising enzymes in neurons, while Fig. 7B shows the predominance of purine metabolising enzymes in astrocytes.

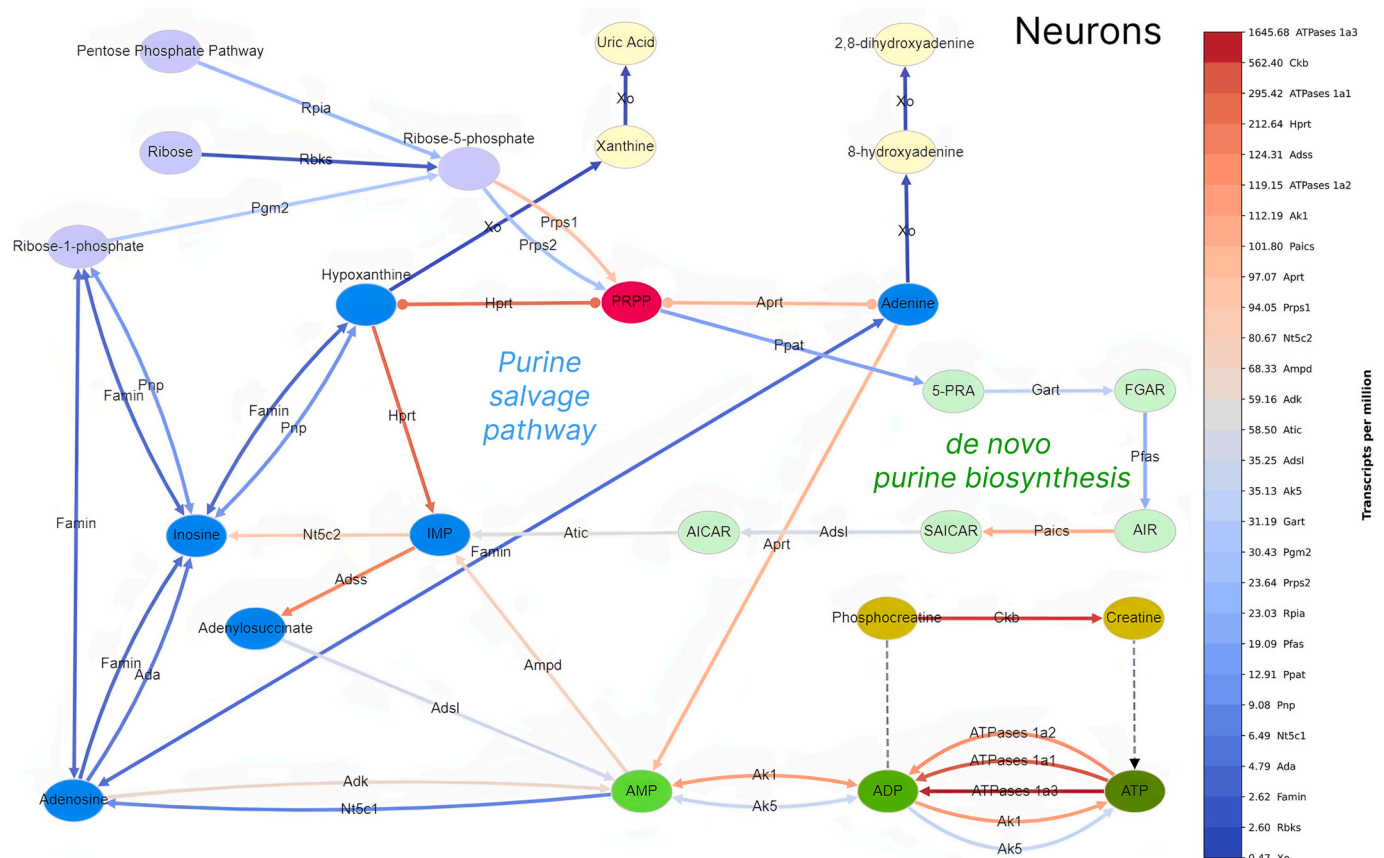


Fig. 5. Purine synthesis and metabolism pathways in neurons. In this and subsequent figures, lilac ellipses represent the entry of ribose into the PSP and DNPB pathway; PRPP is shown in the central red ellipse; the DNPB substrates are displayed in the light green ellipses, while those of the PSP in blue ellipses. The synthesis of adenine nucleotides (AMP → ADP → ATP) is shown in increasing tones of green. Non salvageable metabolites (xanthine and uric acid, 8-hydroxyadenine and 2,8-dihydroxyadenine) for xanthine and adenine, respectively, are shown in yellow ellipses. Round-end connector lines connect PRPP to Aprt and Hppt to indicate its requirement by these enzymes. The arrows are colour-coded from light gene expression (blue), towards high expression (dark red). A maximal threshold was implemented at 300 TPM to have the best possible colour representation despite differences in values. The same threshold was applied to astrocytes to allow better comparability. Notable in neurons are the higher expression levels of enzymes involved in adenine nucleotide synthesis, HPRT, APRT and the adenylate kinases (AK1, AK5), compared to astrocytes (Fig. 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Purines as fundamental biochemical moieties

Purine-based molecules perform a wide range of genetic and biochemical tasks that are fundamental to life. These include in RNA, DNA, intracellular (cGMP, cAMP) and extracellular signaling molecules (ATP, ADP, adenosine, inosine), and in the universal energy currency of ATP. Their potential seeding, and that of the pyrimidines and ribose from extra-terrestrial sources (eg via meteorites) during the early development of the Earth (Hayatsu, 1964; Callahan et al., 2011; Furukawa et al., 2019; Oba et al., 2022) may have allowed them to occupy such fundamental and ubiquitous niches. It is thus not surprising that a wide array of intra- and extracellular enzymes have arisen to both synthesize and metabolise purine molecules, and for cell surface proteins to have evolved to respond to them. Indeed, the relationship between ATP as an energy source, and its metabolites in helping preserve ATP is a case in point: the intracellular accumulation of ATP metabolites ADP and AMP is sensed by AMP-activated protein kinase (AMPK), which, when activated, including by DNPB pathway intermediary AICAR (5-aminoimidazole-4-carboxamide ribonucleotide; ZMP) promotes alternative energy-generating pathways such as fatty acid oxidation, glycolysis and glucose uptake, while inhibiting energy-consuming pathways such as protein synthesis (Camici et al., 2018; Hardie, 2018). Further degradation to adenosine yields a powerful

extracellular inhibitory neuromodulator capable of suppressing energy-expensive presynaptic neurotransmitter release and postsynaptic neuronal firing (Lezmy et al., 2021), primarily via the adenosine A₁ receptor, but additionally via increasing nutrient supply through adenosine A₂ receptor-mediated vasodilation (Frenguelli and Dale, 2020). Similarly, FAMIN, possessing adenosine deaminase, purine nucleoside phosphorylase, S-methyl-5'-thioadenosine phosphorylase, and previously undocumented eukaryotic adenosine phosphorylase activity, may represent an early multi-functional mechanism to regulate the purine nucleotide cycle in cells (Cader et al., 2020).

4.2. Inborn errors of purine metabolism

Given the fundamental role of purines in many biological processes, it is not surprising that mutations in key purine pathway enzymes lead to a wide range of neurological and other disorders (Dewulf et al., 2021; Garcia-Gil et al., 2021; Jurecka and Tytki-Szymanska, 2022). These include deficiencies in ADSL (OMIM 103050; autism, epilepsy, psychomotor delay); ATIC (OMIM 608688; neurodevelopmental impairment, blindness, epilepsy); HPRT (OMIM 300322, Lesch-Nyhan syndrome; neurodevelopmental impairment, dystonia, chorea, self-mutilating behaviour); PNP (OMIM 613179; motor impairment, cognitive/behavioural impairment) and PRPS1 (OMIM 301835, Arts Syndrome; OMIM 311070, Charcot-Marie-Tooth disease-5; OMIM 304500, X-linked deafness-1; mental retardation, delayed and impaired

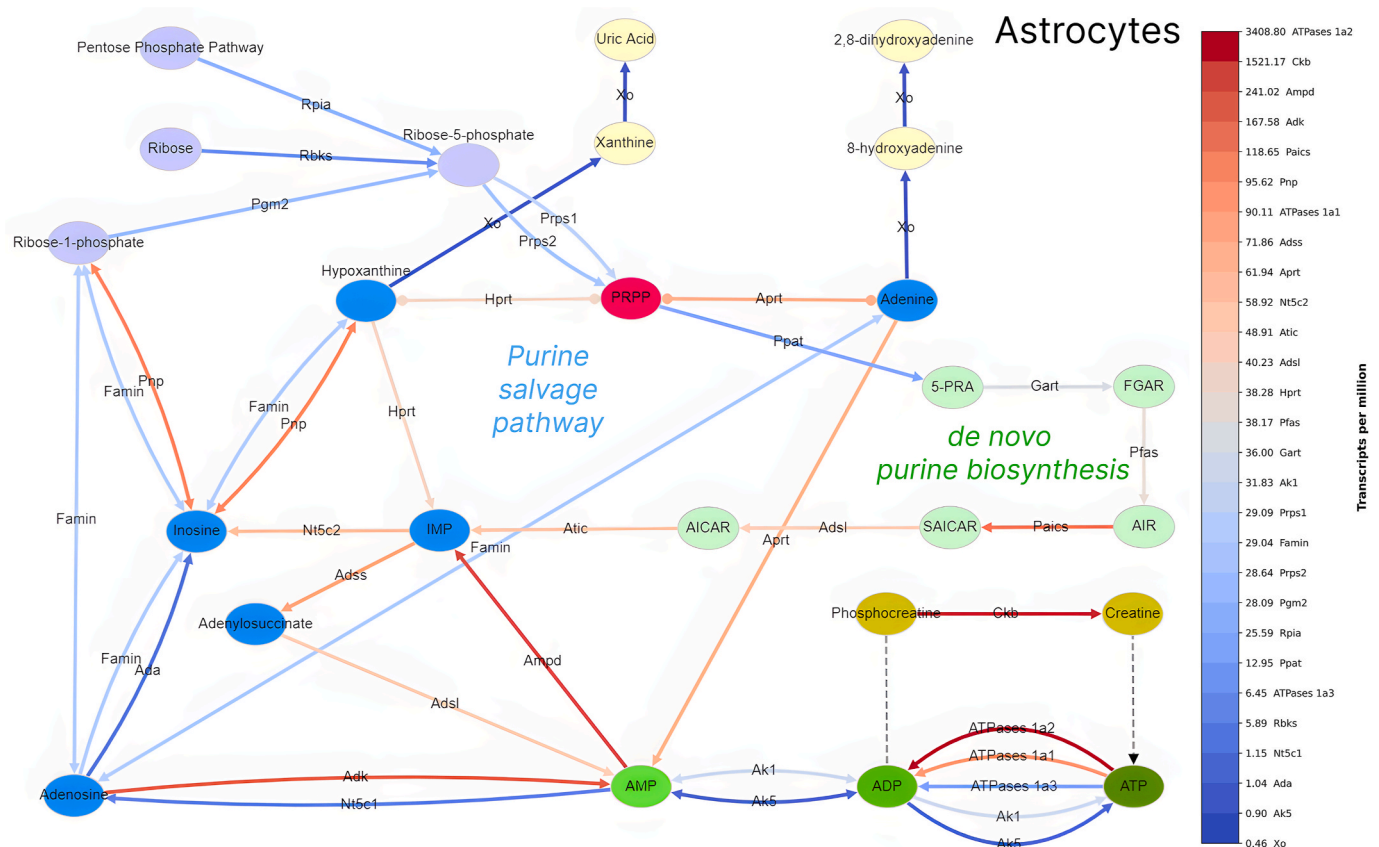


Fig. 6. Purine synthesis and metabolism pathways in astrocytes. Colour scheme and TPM maximum threshold (300) as per Fig. 5. Notable are the strong expression levels of purine metabolising enzymes AMPD and PNP.

motor development, hearing and visual impairment). In the case of the recently-described mutations in PAICS (OMIM 619859) these lead to multiple congenital malformations, purinosome absence and early postnatal death (Pelet et al., 2019). In somewhat stark contrast, a deficiency in APRT (OMIM 614723) does not lead to obvious neurological impairment, but instead to urinary stones due to the accumulation in the kidney of the insoluble adenine metabolite 2,8-dihydroxyadenine (DHA; Figs. 5–7). This condition can be successfully treated with a xanthine oxidase inhibitor, such as allopurinol or febuxostat, which prevents the xanthine oxidase-mediated conversion of adenine to DHA (Edvardsson et al., 2017). Perhaps equally surprising, given FAMIN's multifunctional role, is the apparent lack of neurological manifestations of mutations in FAMIN (OMIM 613409) with juvenile idiopathic arthritis and other inflammatory conditions being most frequently observed (Cader et al., 2020).

4.3. Expression of purine synthesis and metabolising enzymes in neurons and astrocytes

Our analysis of an RNA-Seq database of the purine synthesis and metabolising enzymes of early post-natal (P7) murine cerebral cortex neurons and astrocytes (Zhang et al., 2014) revealed appreciable differences between the PSP and DNPB pathway, and between neurons and astrocytes. That the cell types were appropriately segregated, in addition to the evidence provided in the original publication (Zhang et al., 2014), is further evidenced by the appropriate high expression of the neuronal Na^+/K^+ ATPase $\alpha 3$ subunit, and the astrocytic Na^+/K^+ ATPase $\alpha 2$ subunit (Shrivastava et al., 2020). Similarly, adenosine kinase (Adk), an enzyme predominately localised in astrocytes (Studer et al., 2006; Etherington et al., 2009), was expressed at ~ 3 -fold higher levels in the astrocyte population. Thus, at least at this developmental time-point, the

transcriptomic data provides a reliable account of the expression levels of enzymes pertinent to the synthesis and metabolism of ATP in astrocytes and neurons. Moreover, the expression levels of the genes considered here exceed the values described by Zhang and colleagues (Zhang et al., 2014) as being statistically significant.

DNPB enzymes were generally expressed at low levels in both neurons and astrocytes, except for PAICS, which was expressed at relatively high levels in both cell types. This would suggest, as has been proposed previously (Gerlach et al., 1971; Allsop and Watts, 1980; Barsotti et al., 2002; Pareek et al., 2021), that the DNPB does not feature prominently in adenine nucleotide synthesis in the mammalian brain, and less so as development proceeds (Allsop and Watts, 1980). In contrast, the two core PSP enzymes - HPRT and APRT - were expressed at high levels in both neurons and astrocytes, as were the enzymes in both the pathways leading to the production of substrates for the PSP (astrocytes > neurons), and enzymes promoting the synthesis of ATP (neurons > astrocytes). Creatine kinase mRNA was very highly expressed in both cell types, as has been observed previously with in situ hybridization (Tachikawa et al., 2004, 2018). This suggests that phosphocreatine is an important buffer against ATP depletion in both neurons and astrocytes.

4.4. Differential expression of PSP enzymes in neurons and astrocytes

PSP and related enzymes were differentially expressed between neurons and astrocytes to varying degrees. Notably neurons, where APRT and HPRT were respectively expressed 1.6 and 5.6-fold higher than in astrocytes, expressed higher levels of adenylate kinase isoforms AK1 and AK5 than astrocytes (by 3.5- and 39-fold, respectively), with very low levels of AK5 in astrocytes. This suggests both a greater reliance of neurons on the PSP, and, via the adenylate kinases the potential to “trap” AMP and ADP and, together with creatine kinase, for subsequent

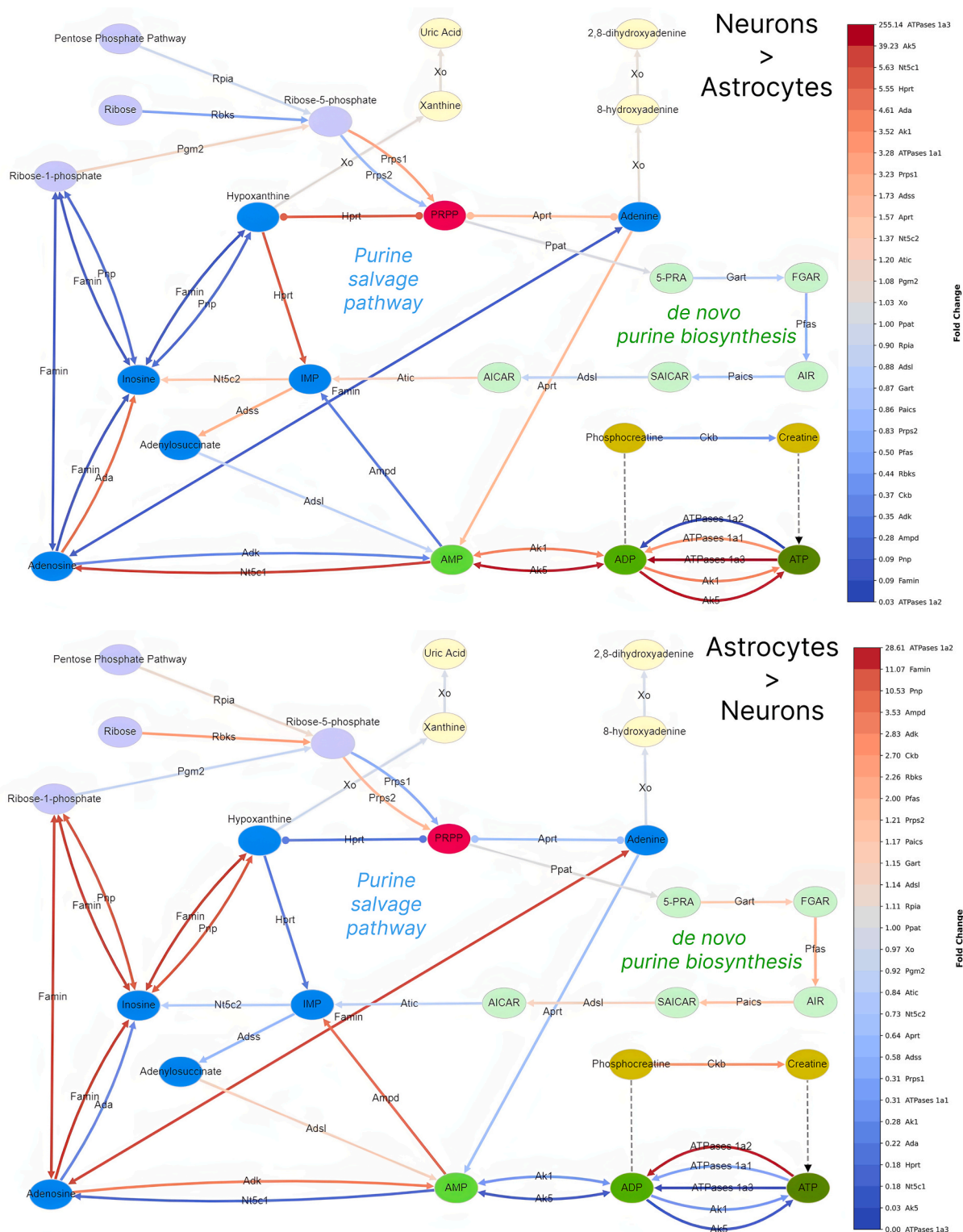


Fig. 7. Differential expression (fold differences) of PSP and DNPB genes in neurons and astrocytes. Colour scheme as per Fig. 5. Top panel: fold differences in gene expression where neurons > astrocytes; arrows with red colours indicate greater expression in neurons and those with blue colours indicate lower expression in neurons compared to astrocytes. Lower panel: fold differences in gene expression where astrocytes > neurons; arrows with red colours indicate greater expression in astrocytes and those with blue colours indicate lower expression in astrocytes compared to neurons. Scale bars provide actual fold difference between cell types. Maximum threshold has been set to 40-fold for both cell types. Note in neurons ATP synthesis pathways predominate (HPRT, APRT, AK1, AK5) whereas in astrocytes, metabolism predominates (AMPD, FAMIN, PNP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conversion to ATP. The adenylate kinases are cytosolic enzymes and possess both nucleoside monophosphate kinase and nucleoside diphosphate kinase activity making them capable of converting AMP to ATP (Panayiotou et al., 2014). Importantly, AK1 and AK5, in addition to using ATP as a phosphate donor, can utilise either GTP (AK5) or any nucleoside triphosphate (AK1) as a source of phosphate (Panayiotou et al., 2014), as well as nucleoside diphosphates (Amiri et al., 2013). This avoids the futile situation where ATP is consumed to generate ATP, and suggests the ability of neurons in particular to synthesize ATP from AMP, the primary product of the PSP.

The emphasis on neuronal ATP synthesis contrasts with the seeming higher expression in astrocytes of enzymes capable of metabolising purines. Notably these included AMPD (AMP \rightarrow IMP) and PNP (inosine \rightarrow hypoxanthine + Rib-1-phosphate). Of the three AMPD isoforms (Camici et al., 2018), AMPD2 and AMPD3 were expressed at 2.6 and 9-fold higher levels in astrocytes than in neurons. In this regard, the fate of IMP in both astrocytes and neurons is unclear from a gene expression analysis alone; IMP is preferentially produced by HPRT in neurons, and by AMPD in astrocytes. Persistence in the purine salvage pathway is promoted by high levels of ADSS in neurons and astrocytes (1.7-fold greater in neurons), but reduced by the presence of NT5C2 in both (1.4-fold higher in neurons). Experimental studies using isotopically-labelled hypoxanthine and adenine, in the presence and absence of xanthine oxidase inhibitors, are required to appreciate the relative incorporation of these two lynchpins of the PSP into adenine nucleotides.

4.5. Implications for targeting the PSP and DNPB pathway

Our analysis of enzymes of the PSP and DNPB pathway has a significant limitation in that it only takes into account gene expression. No information is provided on protein levels, or the relevant kinetic parameters that would determine the flux of metabolites through the pathways, and the rate of production of adenine nucleotides through either route. Nonetheless, it does provide an empirical basis for assuming that these pathways are present in neurons and astrocytes, and clearly shows differential gene expression for the PSP and DNPB pathway. Given this, there is potential for their exploitation in conditions characterised by ATP depletion utilising substrates that may be readily incorporated into them.

To this end, exogenous D-ribose, which possesses high solubility across biological membranes (Sacerdote and Szostak, 2005), has been shown to raise the cellular pool of PRPP (Zimmer, 1998) and result in the stimulation of adenine nucleotide synthesis via the PSP (Barsotti and Ipata, 2002). Similarly, adenine, with its SLC29A1 (2-fold higher expression in neurons) and SLC29A2 transporters, is taken up by cells and readily incorporated into adenine nucleotides (Santos et al., 1968). We have previously shown in acutely-prepared brain slices, comprising hippocampus and overlying neocortex, that the reduced levels ATP caused by slice preparation can be increased by the incubation for 1–2 h of brain slices with the combined application of low concentrations of both adenine (50 μ M) and D-ribose (1 mM; “RibAde”). This elevation in cellular ATP did not occur if either adenine or D-ribose were administered in isolation, despite the fact that these experiments were conducted in oxygenated aCSF containing 10 mM D-glucose, which would be expected to support mitochondrial activity (zur Nedden et al., 2011). This suggests that ribose and adenine do not stimulate mitochondria to synthesize additional ATP from existing ATP precursors, but instead that ribose and adenine provide additional ATP precursors for the subsequent synthesis of ATP, either directly through adenylate kinases or via the provision of ADP to mitochondria. Importantly, when the dead edges of the brain slices were taken into account, ATP levels in the viable tissue were almost identical to those reported *in vivo*, and ATP levels remained stable for several hours after removal of RibAde (zur Nedden et al., 2011). Thus, both the PSP and the feedback mechanisms limiting ATP production are in operation in brain slices. Moreover, once ATP levels

are restored, brain slices, and by extension the brain, possess the mechanisms necessary to maintain this new level of ATP.

This is encouraging as it suggests that ATP-depleting brain injuries caused by stroke, cardiac arrest or traumatic brain injury might benefit from the acute or hyper-acute provision of RibAde, with the obligatory phosphate groups being provided via reactions catalysed by adenylate kinases. In this regard, several features of the adenylate kinases lend themselves to this task, even when ATP levels are reduced. Notably, adenylate kinases possess both nucleoside mono- and diphosphate kinase activities (Amiri et al., 2013), and display a broad substrate specificity for the phosphate donor, which includes both nucleoside diphosphates (Amiri et al., 2013) and nucleoside triphosphates (Panayiotou et al., 2014). This ability allows then to act as adenine nucleotide “shuttles” from areas of ATP generation to areas of ATP utilization (Dzeja and Terzic, 2009). Thus, during and after ATP-depleting conditions, when suitable nucleotide donors decline and recover at different rates (Chapman et al., 1981; Onodera et al., 1986; Kinouchi et al., 1990; Lazzarino et al., 2020), the adenylate kinases may generate ATP via a route that depends upon local substrates. This implies that substrate availability and the extent of ATP recovery will depend upon the site and severity of the initial injury (eg core vs penumbra in ischemic stroke). RibAde may thus provide the means for vulnerable brain tissue to support energy-expensive, but vital functions, such as the maintenance of Na⁺/K⁺ ATPase activity to help maintain ionic gradients and avoid injury-induced cerebral oedema.

Indeed, evidence of additional functional and beneficial consequences of this elevated ATP pool arose when RibAde-treated brain slices were challenged by secondary insults frequently seen in the injured brain: hypoxia/ischemia and seizure activity. In the former, removal of oxygen and glucose caused the release of greater quantities of adenosine, which had a greater inhibitory effect of glutamatergic excitatory synaptic transmission (zur Nedden et al., 2014). In the latter, in RibAde-treated brain slices, seizure activity provoked greater adenosine release, which resulted in reduced seizure burden (Hall and Frenguelli, 2018). These mechanistic observations likely contributed to the encouraging reduction in lesion volume and improved functional recovery observed in RibAde-treated rats after temporary occlusion of the middle cerebral artery, effects that were further enhanced by inclusion of the xanthine oxidase inhibitor allopurinol (Faller et al., 2017).

Thus, by facilitating the restoration of cellular ATP in the injured brain, RibAde could potentially: i) allow neurons and astrocytes to better maintain ionic gradients across membranes, reducing both cellular oedema and the initiation and consequences of harmful cortical spreading depolarisation, and ii) result in greater release of adenosine, resulting in the inhibition of glutamate release and membrane hyperpolarisation, which would likely reduce the frequency or intensity of seizure activity and cortical spreading depolarisations and the energy demands on, and incorporation into the lesion of vulnerable adjacent brain tissue. The inclusion of allopurinol (“RibAdeAll”) would be expected to enhance the actions of RibAde through greater availability of hypoxanthine and adenine for salvage and synthesis into ATP, together with reduced production of damaging hydrogen peroxide-derived reactive oxygen species. The existing use of ribose, adenine and allopurinol in humans (Frenguelli, 2019) suggests that targeting the PSP could be a viable option in the rapid, point-of-injury treatment of acute brain injury.

5. Conclusions

We have shown, using a publicly-available RNA-Seq data set (Zhang et al., 2014), that enzymes of the PSP and DNPB pathway are present in neurons and astrocytes. The lower expression levels of the DNPB pathway compared to the PSP suggest that latter dominates in brain tissue. Moreover, the profile of PSP enzyme expression appears to differ between neurons and astrocytes, with neurons seeming to promote ATP synthesis and astrocytes favouring purine metabolism. Empirical

evidence in brain slices suggests that substrates for the PSP (adenine and ribose) can be utilized by brain tissue to elevate cellular ATP, and that this has functional consequences, not least of which through greater release of the neuroprotective, anticonvulsant and vasodilator adenosine. Exploiting the PSP through compounds already in use in the clinic (adenine, ribose and allopurinol), could therefore give rise to simple, readily-deployable treatments for the rapid treatment of acute brain injury by first responders at the point of injury.

CRediT authorship contribution statement

Philipp Gessner: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Jenni Lum:** Investigation, Data curation, Visualization. **Bruno G. Frenguelli:** Conceptualization, Methodology, Validation, Investigation, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of competing interest

BGF is the Editor-in-Chief of *Neuropharmacology*.

Data availability

Data will be made available on request.

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