Untargeted metabolomics to evaluate polymyxin B toxicodynamics following direct intracerebroventricular administration into the rat brain

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Abstract
There is a dearth of studies focused on understanding pharmacokinetics, pharmacodynamics and toxicodynamics of polymyxins following direct administration to the central nervous system (CNS). In this study, for the first time, untargeted metabolomics were employed to ascertain the perturbations of brain metabolism in the rat cerebral cortex following direct brain injection of 0.75 mg/kg polymyxin B (1 and 4 h) through the right lateral ventricle. In the right cortex metabolome, ICV polymyxin B induced a greater perturbation at 1 h compared to negligible effect at 4 h. Pathway enrichment analysis showed that sphingolipid, arginine, and histidine metabolism, together with aminoacyl-tRNA biosynthesis were significantly affected in the right cortex metabolome. Furthermore, intracerebroventricular (ICV) polymyxin B dysregulated the two arms (CDP-choline and CDP-ethanolamine) of the Kennedy pathway that governs the de novo biosynthesis of neuronal phospholipids. Importantly, the key intermediates of metabolic pathways that maintain cellular redox balance (e.g., glutathione metabolism) and mitochondrial function (e.g., electron transport chain) were markedly depleted. The abundance of key metabolites (e.g., N-acetyl-L-glutamate) associated with diverse CNS disorders (e.g., neurodegenerative disease) were also significantly perturbed. The biological significance of these metabolic perturbations on the CNS includes impaired oxidant-antioxidant balance, impaired neuronal lipid homeostasis and mitochondrial dysfunction. Furthermore, ICV polymyxin B caused a significant alteration in the abundance of several metabolic biomarkers associated with cerebral ischemia, oxidative stress as well as certain neurological disorders. These findings may facilitate the development of new pharmacokinetic/pharmacodynamic strategies to attenuate polymyxin ICV related CNS toxicities and stimulate the discovery of safer next-generation polymyxin-like lipopeptide antibiotics.

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1. Introduction

The world is poised to enter the post-antibiotics era, where common infections and minor injuries could prove to be lethal and unresponsive to conventional antibiotics [1]. Gram-negative bacteria that cause life-threatening infections, such as Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii, have developed resistance to almost all currently approved antibiotics [2,3]. Polymyxins are used as last resort antibiotics, largely owing to their efficacy against multi-drug resistant (MDR) Gram-negative bacteria [4].

The clinically available polymyxins, namely polymyxin B and E (syn. colistin) are a group of cationic cyclic lipo-decapeptides that were introduced into the market in the 1950s [3]. Polymyxin use in the clinic waned in the 1970s due to the frequent incidence of nephrotoxicity and neurotoxicity [4,5]. However, their utilization has steadily increased in recent times for the treatment of sepsis and CNS infections caused by Gram-negative ‘superbugs’; given the limited alternative therapeutic options [6].

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There are three critical barriers facing the safer and more efficacious use of polymyxins for the treatment of CNS infections, first and foremost they have a narrow therapeutic window. Second, our understanding of the mechanisms of polymyxin neurotoxicity in the CNS is lacking. Finally, there is a dearth of studies focused on understanding pharmacokinetics, pharmacodynamics and toxicodynamics of polymyxins following direct CNS administration [4,7,8]. Unfortunately, parenteral administration of polymyxins is ineffective for the treatment of CNS infections as only a small fraction of the administered drug can reach the site of infection due to the poor blood–brain barrier (BBB) permeability [9,10]. As a consequence, polymyxin exposure at the site of infection within the CNS is suboptimal which results in poor efficacy and potentially promotes the emergence of resistance. Reportedly, dose escalation of intravenously administered polymyxins to achieve higher exposure within the CNS is more likely to be associated with high systemic exposure resulting in severe nephrotoxicity [11,12].

Several invasive techniques have been developed to bypass the BBB and ease the delivery of antibiotics directly into the brain tissue, including intrathecal (ITH, via the lumbar cistern of the spinal canal) or intracerebroventricular (ICV, via insertion of a catheter into the lateral ventricle of the brain) [13,14]. Based on clinical experience, it has been shown that ITH/ICV colistin methanesulphonate (CMS; the prodrug of colistin) and polymyxin B are the most effective therapeutic options for the treatment of MDR Gram-negative CNS infections [15–18]. However, given that even normal daily doses (5 to 7 mg/kg/day) of parenteral polymyxins can cause neurological side effects (e.g., vasomotor instability, ataxia, dizziness, convulsions of varying severity, and apnea) in cystic fibrosis patients [19], these routes of delivery (ITH/ICV) are not without drawbacks, including neurological adverse effects. Several experimental studies with animals (e.g., rabbits, cats, dogs and monkeys) showed that ITH/ICV polymyxin delivery associated with neurological complications and death [20–23]. Notably, polymyxins are equally toxic to human CNS tissue as similar neurological complications such as seizures, hypotonia and cauda equine syndrome were also reported in patients with CNS infections following ITH/ICV CMS and polymyxin B using currently recommended dosage regimens [15,24–27]. Therefore, the polymyxin exposure levels need to be optimized to achieve the right balance between maximizing the desired antibacterial effect while minimizing the potential for neurotoxicity. Unfortunately, current dosage recommendations for ITH/ICV polymyxins are empirical in nature due to the lack of pharmacological data, which significantly hinders their clinical usefulness [17]. To date, our understanding of the precise mechanisms of polymyxin-induced neurotoxicity and disposition in CNS tissues remains largely unknown.

Metabolomics is a cutting-edge technique with wide-spread applications across drug discovery, microbial biotechnology, toxicology and the identification of biomarkers for disease diagnosis and risk prediction [28,29]. This rapidly emerging field combines different strategies to identify, quantify, and profile metabolites and small molecules in various biological samples such as cells, tissues, organisms, and biofluids [30,31]. In previous studies metabolomics has been successfully used as an approach to give a holistic insight into the complex mechanisms of drug induced neurotoxicity [30,32,33]. Undeniably, a thorough understanding of the underlyings mechanisms of polymyxin B neurotoxicity in the CNS will allow for the rational design of polymyxin treatment regimens that will result in safer polymyxin B exposure profiles well within the narrow therapeutic window with improved treatment outcomes for CNS infections. This study represents the first report aimed at elucidating the neurotoxicity of polymyxin B in the CNS using untargeted metabolomics following ICV administration of a therapeutically relevant dose into the rat brain. The study provides the first fundamental understanding of the metabolic neurotoxic mechanisms associated with ICV polymyxin B therapy and could facilitate optimisation of clinical dosage regimens to avoid polymyxin neurotoxicity.

2. Results and discussion

2.1. Metabolite profiling of the right and left cerebral cortex following ICV polymyxin B treatment

ICV polymyxin B treatment of rats induced significantly more perturbations in the right cortex metabolome compared to the left cortex, understandably as we directly delivered the dose into the right lateral ventricle as we have previously reported in great detail [34]. A total of 1018 putatively metabolites were acquired, which included lipids (21.7 %), peptides (10.5 %), amino acids (9.9 %), carbohydrates (6.8 %) and nucleotides (4.3 %) (Supplementary Fig. S1). Principal component analysis (PCA) demonstrated that ICV polymyxin B induced a greater number of changes in the metabolome of the right cortex at 1 h, as manifested by the well-separated treatment and control groups (Supplementary Fig. S2A). However, a significant overlap was evident at 4 h between the ICV polymyxin B treated and untreated control groups, suggesting that the effects of polymyxin B exposure were wearing off on the right cortex with time (Supplementary Fig. S2A). The PCA plots for the left cortex demonstrated that the polymyxin B treated samples were indistinguishable from the untreated control group at 1 h, whereas at 4 h the two groups diverged, indicative of a differential effect of ICV polymyxin B on the left cerebral cortex metabolome over time compared to the untreated control (Supplementary Fig. S2B). Polymyxin B primarily accumulated in the right cerebral cortex and produced a greater global metabolome perturbation in this region (Supplementary Table S2). Notably, the observed differential time effects of ICV polymyxin B on both hemispheres reflects a time-dependent biodistribution for polymyxin B, which certainly warrants further PK/PD studies. In line with the PCA plots, the heatmap of the right cortex metabolome demonstrated extensive changes in the ICV polymyxin B treated group in comparison to the untreated control at 1 h; while far less perturbations were evident at 4 h (Supplementary Fig. S3). While the heatmap for the left cortex indicated the opposite, ICV polymyxin B had negligible impact on the left cerebral cortex metabolome at 1 h; however, significant perturbations were evident at 4 h (Supplementary Fig. S3).

Our results showed significant differences in 345 (all decreased) and 7 (4 increased, 3 decreased) metabolites in the metabolome of the right cortex at 1 and 4 h, respectively (Supplementary Fig. S4A). ICV polymyxin B induced far less perturbation in the left cortex metabolome at 1 h, with only 5 (2 increased, 3 decreased) significantly perturbed metabolites. Whereas a latent effect was observed at 4 h, as manifested by the perturbation of 77 (20 increased, 57 decreased) metabolites in the left cortex metabolome (Supplementary Fig. S4A).

Among the significantly perturbed metabolites of the right cerebral cortex at 1 h, undefined (109 down) metabolites were the highest number followed by intermediaries such as lipids (102 down), amino acids (49 down) and peptides (35 down) (Supplementary Fig. S4B). The metabolome perturbation profile was the same at 4 h, albeit, the total number of metabolites was far lower. The majority consisted of the undefined (1 down, 2 up) metabolite class followed by lipids (1 down, 1 up), amino acids (1 up), cofactors and vitamins (1 down) (Supplementary Fig. S4B). The undefined, lipids and nucleotide metabolites constituted the main perturbed classes from the left cortex metabolome in response to ICV polymyxin B treatment at 1 h (Supplementary Fig. S4B). A different metabolome perturbation profile was seen in the left cortex
at 4 h wherein intermediaries such as lipids (51 down, 2 up) accounted for the majority of the metabolite classes; followed by undefined (4 down, 8 up) and peptides (2 down, 6 up) (Supplementary Fig. S4B).

2.2. Pathway enrichment analysis for the significantly perturbed metabolites of the right cerebral cortex following ICV polymyxin B treatment

Considering that ICV polymyxin B induced extensive perturbations in the metabolome of the right cortex at 1 h and only a frivolous effect at 4 h, we only mapped and analysed the significantly impacted metabolic features at 1 h (Supplementary Fig. S4A and S4B). The mapping of the significant features of the right cortex at 1 h revealed that sphingolipid biosynthesis (14 intermediates), aminoacyl-tRNA biosynthesis (12 intermediates), glutathione (11 intermediates), taurine and hypotaurine (9 intermediate) and histidine (8 intermediates) metabolism, electron transport chain (6 intermediates) and tricarboxylic acid cycle (6 intermediates), were amongst the most significantly perturbed pathways (Supplementary Fig. S5A; Supplementary Table S1).

2.3. Lipid metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

ICV polymyxin B treatment caused major alterations across all lipid classes predominantly, glycerophospholipids followed by fatty acids (Fig. 1A and 1B). The glycerophospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the major components of the neuronal cellular membrane and play major roles in a wide range of biological processes such as in the functioning of the synapses, regulation of neurotransmitters and lipid signalling [35]. Dysregulation in neuronal phospholipid homeostasis is frequently indicative of post-ischemic neurodegeneration in the CNS [36]. Among the glycerophospholipids, the abundance of CDP-ethanolamine [log2 Fold change (FC) = −2.6] and CDP-choline (log2FC = −2.5) underwent a significant decline following ICV polymyxin B treatment (Fig. 1A). The two arms of the Kennedy pathway CDP-choline and CDP-ethanolamine are essential pathway branches responsible for the de novo biosynthesis of PC and PE, respectively, in mammalian cell membranes, particularly in CNS tissues [37,38]. Notably, ICV polymyxin treatment significantly decreased the levels of two essential omega-3 polyunsaturated fatty acids (PUFAs) namely docosahexaenoic acid (DHA) and docosapentaenoic acid (log2FC ≥−4.0; p < 0.05) (Fig. 1B) [39,40]. It is well-known that DHA plays a key role in the growth and functional development of the brain and its deficiency is associated with poor visual and neural development in humans [41]. Furthermore, it possesses neuroprotective properties by minimising the risk of lifelong neurological impairments resulting from brain injury [42].

Intriguingly, the abundance of polyunsaturated omega-6 fatty acid arachidonic acid (ARA) also markedly declined in response to ICV polymyxin B treatment (log2FC = −5.4) (Fig. 1B). ARA constitutes up to 20 % of fatty acids in the mammalian brain and acts as a retrograde messenger in postsynaptic neurons [43]. Notably, perturbations of ARA metabolism in the brain have been linked to neurological, neurodegenerative, and psychiatric disorders [44].

2.4. Sphingolipid metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

Sphingolipids are ubiquitous in the CNS and exhibit multiple biological functions, including neuronal tissue development, cell signalling and as toxin receptors [45]. Defects in sphingolipid metabolism are strongly correlated with multiple neurodegenerative diseases and may contribute to their complex pathogenesis.
The de novo pathway for sphingolipid biosynthesis begins with the condensation of serine with the activated C16 fatty acid palmitoyl-CoA, which is catalysed by serine palmitoyltransferase (SPT) [47]. ICV polymyxin B treatment caused a significant depletion in the levels of ten fundamental metabolites of the sphingolipid biosynthetic pathway, including palmitoyl-CoA, serine, sphinganine-1-phosphate, sphingosine 1-phosphate (S1P), ceramide, galactosylceramide, sulfatide, ethanolamine phosphate, glucosylceramide and sphingomyelin (SM) (log2FC ≥ −1.5; p < 0.05) (Fig. 2A & B). Four sphingomyelin pathway metabolites experienced a significant decline in their levels following ICV polymyxin B treatment, namely SM(d16:1/22:0), SM(d18:0/22:1), SM(d18:1/24:0) and 3-0-sulfogalactosylceramide (≥−1.5-log2FC; p < 0.05) (Fig. 2B). S1P, a bioactive sphingolipid metabolite, functions as a modulator in neurotransmitter release, synaptic transmission and neuroinflammation [48]. Pharmacological inhibition or genetic ablation of S1P signalling in the CNS leads to neurodegeneration, stroke, and brain tumours [49]. SM is enriched in the CNS and mainly constitutes the myelin sheath that surrounds neuronal axons and therefore maintains myelination integrity and function in the CNS [50].

2.5. Mitochondrial electron transport chain (ETC) and tricarboxylic acid (TCA) cycle metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

ICV polymyxin B treatment caused significant mitochondrial dysfunction as manifested by a significant alteration in the abundance of six key intermediates involved in the ETC, including ATP (log2FC = −4.2), FAD (log2FC = −6.3), NADH (log2FC = −8.6), flavin mononucleotide (FMN, log2FC = −4.7), orthophosphate (log2FC = −5.9) and pyrophosphate (log2FC = −9.8) (Fig. 3A and B). Concomitantly, the abundance of six essential metabolites namely 2-oxoglutarate (2-OG), acetyl-CoA, malate, succinate, fumarate, and citrate of the TCA cycle, were significantly decreased following ICV polymyxin B treatment (log2FC ≥−3.0; p < 0.05) (Fig. 3A and B). These findings are consistent with our previous study which showed that intravenous administration of colistin attenuated the mitochondrial respiratory chain activity in sciatic nerve tissues of mice [51,52].

2.6. Glutathione and ferroptosis metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

Reactive oxygen species (ROS) are continuously produced at high rates within the brain and if cumulative effects ensue, ROS has been reported to cause cerebral tissue damage, neuroinflammation, and ultimately neuronal death [53]. One of the main protective mechanisms of the cellular ROS detoxification system is the glutathione (GSH) antioxidant system [54]. Notably, ICV polymyxin B treatment induced a significant depletion in the abundance of eleven essential precursors of glutathione metabolism, including reduced glutathione (GSH), oxidized glutathione (GSSG), l-cysteine, glutamate, γ-glutamylcysteine, l-cysteinylglycine, NADPH, acetyl-CoA, dehydroascorbic acid, ornithine and pyrogallamic acid (log2FC ≥−1.0; p < 0.05) (Fig. 4A). Purportedly, the disruption of oxidant-antioxidant balance within the brain leads to glutathione depletion, which simultaneously induces cerebral biochemical impairment and ultimately manifests into neurodegenerative disorders [55]. Our group has previously reported that polymyxin treatment causes a significant decrease in the levels of GSH in N2a neuronal and A549 lung epithelial cells, which occurs concomitantly with an increase in the levels of intracellular ROS [56,57]. Perturbations in the ferroptosis pathway were also evident in the right cortex metabolome following ICV polymyxin B treatment. These manifested via a significant decline in the abundance of nine key metabolites of the ferroptosis pathway, namely arachidonic acid (log2FC = −5.2), adrenic acid (log2FC = −5.7), GSH (log2FC = −9.0), GSSG (log2FC = −5.2), coenzyme A (log2FC = −5.2), acetyl-CoA (log2FC = −3.8), l-cysteine (log2FC = −5.7), γ-glutamylcysteine (log2FC = −2.8), (R) 2,3-dihydroxy-3-methylvalerate (log2FC = −1.1) and glutamate (log2FC = −1.8) (Fig. 4A). The ferroptosis pathway mediates a form of programmed neuronal cell death that is characterized by the iron-dependent accumulation of lipid peroxides and glutathione consumption [58]. It has been reported that there is a strong connection between acute CNS injuries (e.g., stroke, traumatic brain injury and spinal cord injury) and ferroptosis [58,59]. Notably, this pathway has been investigated as a druggable target for the treatment of complications following acute CNS injuries and a few promising neuroprotective agents have been identified (e.g., ferrostatin-1 and

![Fig. 2. Sphingolipid pathway. (A) Schematic diagram depicting the significantly perturbed sphingolipid pathway metabolites in the right cerebral cortex metabolome following ICV polymyxin B treatment. Attenuated metabolites are highlighted by blue rectangles. (B) Fold changes for the significantly impacted metabolites of the sphingolipid pathway and sphingomyelins after ICV polymyxin B treatment. (log2FC ≥−1.5; p < 0.05). Blue rectangle: attenuated metabolites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
liproxstatin-1) which may also be amenable as neuroprotective co-therapeutics during CNS polymyxin treatment [59,60].

2.7. Arginine pathway metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

Arginine metabolism in the brain is essential for the production of a diverse array of neurotransmitters, such as l-citrulline and nitric oxide (NO) which are derived from l-arginine via the action of nitric oxide synthase (NOS) [61]. NO, usually produced by neurons and endothelial NOS, plays a crucial role in maintaining cerebral blood flow; however, excessive NO production can lead to neurotoxicity [62]. Conversely, suboptimal levels of NO due to impairments in arginine metabolism within the brain gives rise to production of inimical peroxynitrite which can also lead to neuronal cell death [63]. We observed that the levels of eight essential precursors of arginine metabolism were significantly perturbed in the right cortex metabolome after ICV polymyxin B treatment, namely L-arginine (log₂FC = 1.24), glutamate (log₂FC = 1.82), L-ornithine (log₂FC = 4.2), guanidoacetic acid (log₂FC = 7.2), creatine (log₂FC = 13.6), γ-glutamylcysteine (log₂FC = 7.2), L-lysine (log₂FC = 4.8), and L-proline (log₂FC = 7.2). These observations highlight the importance of arginine metabolism in the context of polymyxin B-induced neurotoxicity.
2.8. Histidine pathway metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

Histidine plays a major neuroprotective role in astrocytes during the early and late stages of cerebral ischemia [64]. Furthermore, it has been reported that histidine supplementation can have neuroprotective effects against epileptic seizures, in a dose-dependent manner [65]. ICV polymyxin B treatment significantly perturbed histidine metabolism in the right cerebral cortex (Fig. 4B). The abundance of eight principal metabolites of the histidine pathway underwent a significant depletion in the right cerebral cortex metabolome namely histidine, log2FC = −6.6; carnosine, log2FC = −4.7; 4-imidazolone-5-propionic acid, log2FC = −4.3; oxoglutaric acid, log2FC = −4.3; hydantoin-5-propionic acid, log2FC = −4.0; 1-methylhistamine, log2FC = −3.6; N-formimino-1-glutamate, log2FC = −3.5; glutamate log2FC = −1.8 (Fig. 4B).

2.9. Taurine and hypotaurine metabolite perturbations of the right cerebral cortex following ICV polymyxin B treatment

Taurine is one of the most ubiquitous amino acids in the brain and has several pharmacological actions including neuromodulation, immunomodulation and antioxidant effects [66]. Taurine biosynthesis in the brain involves the metabolic cooperation between astrocytes and neurons [66]. Taurine deficiency is associated with brain development abnormalities and neurodegenerative diseases [67]. ICV polymyxin B treatment markedly disrupted taurine and hypotaurine metabolism in the right cerebral cortex metabolome wherein a dramatic decrease in the levels of nine essential metabolites were observed, namely taurine, hypotaurine, taurocyamine, L-cysteine, acetyl-CoA, glutamate, oxoglutaric acid, L-cysteic acid and sulfoacetate (log2FC ≥ −1.0; p < 0.05) (Fig. 5A and B). These findings are in line with a previous untargeted metabolomics study conducted by our group which investigated the mechanisms of polymyxin-induced pulmonary toxicity [68], wherein it was shown that polymyxin B induced marked perturbations of the taurine-hypotaurine pathway in A549 lung epithelial cells.

2.10. Aminoacyl-tRNA biosynthetic pathway perturbations of the right cerebral cortex following ICV polymyxin B treatment

The breakdown of biological processes mediated by aminoacyl-tRNA synthetases (AaRSs) is associated with CNS disorders [69]. It has been found that the inactivating mutations in lysyl-tRNA synthetase and QARS (encoding glutaminyl-tRNA synthetase) have been associated with various CNS impairments, including seizures, hypotonia and ataxia [70,71]. Perturbations in the aminoacyl-tRNA biosynthetic pathway were observed in the right cerebral cortex metabolome following ICV polymyxin B treatment (Table 1). This was reflected by the significant decrease in the abundance of twelve key metabolites of aminoacyl-tRNA biosynthesis, namely L-glutamate, L-glutamine, L-asparagine, L-lysine, L-phenylalanine, L-proline, L-tryptophan, L-arginine, L-methionine, L-histidine, L-valine and L-cysteine (log2FC ≥ −1.0; p < 0.05) (Table 1).

2.11. Analysis of perturbed biomarkers involved in various CNS disorders

Eighteen diverse biomarkers involved in various CNS disorders were significantly impacted after ICV polymyxin B treatment in the right cerebral cortex at 1 h (Table 2). These included endogenous neurotransmitters (glutamate, log2FC = −1.7; N-acetyl-L-glutamate (NAG), log2FC = −8.6; glutamine, log2FC = −1.4) that activate N-methyl-D-aspartate (NMDA) receptors, which in turn promote neuroprotection against excitotoxic insults, oxidative stress and apoptosis (Table 2) [72,73]. Others included essential intermediates involved in oxidative stress responses, such as carnosine (log2FC = −4.7), homocarnosine (log2FC = −9.3) and (R)-3-hydroxybutanoate (3-OH-C4, log2FC = −2.2) (Table 2). Furthermore, the abundance of three fundamental intermediates associated with neurological disorders, such as epilepsy and seizures, were significantly decreased, namely L-pipecolate, a by-product of the

![Fig. 5. Taurine-hypotaurine pathway. (A) Diagrammatic representations and (B) bar charts for significantly perturbed metabolites of the taurine-hypotaurine pathway in the right cerebral cortex metabolome following ICV polymyxin B treatment at 1 h (log2FC ≥ −1.0; p < 0.05). Attenuated metabolites are highlighted by blue rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
Table 1
List of significantly impacted metabolites of aminoacyl-tRNA biosynthetic pathway in the right cerebral cortex metabolome following ICV polymyxin B therapy (log2FC; p < 0.05). The table includes the fold change, significance of change, p-value, aminoacyl tRNA and aminoacyl tRNA synthetase.

| Cognate amino acid | p-value | Log2(FC) | Aminoacyl tRNA | Aminoacyl tRNA synthetase |
|-------------------|---------|----------|----------------|--------------------------|
| L-Arginine        | 0.0004  | -1.2     | L-Arginyl-tRNA | Arginine-tRNA ligase      |
| L-Lysine          | 0.0036  | -1.4     | L-Lysyl-tRNA   | Lysine-tRNA ligase        |
| L-Proline         | 0.0039  | -3.8     | L-Prolyl-tRNA  | Proline-tRNA ligase       |
| L-Phenylalanine   | 0.0052  | -9.9     | L-Phenylalanyl-tRNA | Phenylalanine-tRNA ligase |
| L-Tryptophan      | 0.0013  | -5.7     | L-Tryptophanyl-tRNA | Tryptophan-tRNA ligase   |
| L-Glutamine       | 0.0035  | -1.4     | L-Glutaminyl-tRNA | Glutaminyl-tRNA ligase    |
| L-Glutamate       | 0.0029  | -1.7     | L-Glutamyl-tRNA | Glutamyl-tRNA reductase   |
| L-Asparagine      | 0.0042  | -1.2     | L-Asparaginyl-tRNA | Asparagine-tRNA ligase  |
| L-Cysteine        | 0.0046  | -5.7     | L-Cysteinyl-tRNA | Cysteine-tRNA synthase   |
| L-Methionine      | 0.0066  | -9.5     | L-Methionyl-tRNA | Methionine-tRNA ligase    |
| L-Histidine       | 0.0016  | -6.6     | L-Histidyl-tRNA | Histidine-tRNA ligase     |
| L-Valine          | 0.0196  | -6.7     | L-Valyl-tRNA   | Valine-tRNA ligase        |

Table 2
List of significantly altered biomarkers involved in various CNS disorders of the right cerebral cortex metabolome after ICV polymyxin B treatment at 1h (≥1.0-log2FC; p < 0.05).

| Metabolite                  | Log2FC | Note                                                                 |
|-----------------------------|--------|----------------------------------------------------------------------|
| Glutamate                   | -1.7   | Glutamate is a naturally occurring neurotransmitter that is distributed in over 90 % of all brain synapses [84]. Abnormalities of glutamatergic neurotransmission is believed to cause multiple CNS disorders, including some degenerative brain diseases, mental exhaustion and concentration problems [85]. |
| N-acetyl-L-glutamate (NAG)  | -8.6   | NAG is a peptidase and the third-most-prevalent neurotransmitter in the central nervous system. It activates NMDA receptors and inhibits synaptic release of GABA from cortical neurons [73]. |
| Glutamine                   | -1.4   | Glutamine is ubiquitous in the CNS where it involves in a variety of metabolic pathways such as the generation of both excitatory and inhibitory neurotransmitters (glutamate and γ-aminobutyric acid) [84]. |
| Carnosine                   | -4.7   | Carnosine helps scavenge reactive oxygen species (ROS) during oxidative stress and prevents brain damage [86]. It has also anti-ischemic properties in cerebral ischemia [87]. |
| Homocarnosine               | -9.3   | Homocarnosine is a dipeptide derived from GABA and histidine in brain [88]. It plays multiple physiological role in brain function such as an endogenous antioxidant, neuromodulator, and neuroprotective molecule [89]. |
| (R)-3-hydroxybutanoate      | -2.2   | β-Hydroxybutyrate (JOHB) is involved in cerebral ketone metabolism which is mediated by oxidative stress. It protects neurons against excitotoxicity and oxidative stress [90]. |
| L-Pipecolate                | -1.9   | L-pipecolate is a by-product of pipecolate pathway and L-lysine metabolism in human brain. An increase in L-pipecolate levels is associated with pyridoxine-dependent epilepsy [76]. |
| L-Proline                   | -3.8   | L-proline is distributed profusely in the central nervous system and structurally is a GABA-mimetic. Alteration in L-proline levels impairs GABA production and ultimately, leads to neuropsychiatric disorders [91]. |
| L-Lysine                    | -1.4   | Cerebral L-lysine degradation is the leading cause of pyridoxine-dependent epilepsy and seizure [74]. |
| Docosahexaenoic acid (DHA)  | -4.4   | It is a neuroprotective agent that helps to minimise the risk of lifelong neurological impairment resulted from brain injury [42]. |
| L-Tryptophan (Trp)          | -5.7   | Trp is an essential precursor of the monoaminergic neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) and the rate of 5-HT biosynthesis is limited by Trp availability [92]. Trp depletion is usually associated with irritability and aggression in human [93]. |
| Gamma-Aminobutyric Acid (GABA) | -9.5  | Naturally existing amino acid that works as an inhibitory neurotransmitter in brain. Lower level of GABA associated with several CNS disorders such as seizures, movement disorders and anxiety [94,95]. |
| Creatine                   | -14.1  | Creatine is essential to produce adenosine triphosphate (ATP), which is needed to maintain high energy levels in the brain. Cerebral creatine deficiency is associated with seizure, movement disorders, and muscle weakness [78]. |
| Acetyl-L-carnitine (ALCAR)  | -2.2   | Acetyl carnitine regulates fatty acid metabolism in brain and improves brain health particularly mitochondrial function, activity of the neurotransmitter acetylcholine, and cognitive vitality [96]. Its depletion is mainly associated with major depressive disorder and cognitive dysfunction [97]. |
| ATP                         | -4.2   | ATP production rate increases in the brain, which leads to improved energy supply, supporting neurotransmitters cycling and sustaining the cell signalling in the brain [77]. |
| Fructose 2,6-biphosphate     | -4.0   | Fructose-1,6-biphosphate protects astrocytes of central nervous system from hypoxic damage [98]. |
| Asparagine                  | -1.2   | Asparagine is involved in development and function of the brain. Its deficiency in the brain has been associated with the neurological impairments and brain damage [99]. |

pipecolate pathway, L-proline (an activator of NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA), and L-lysine (log2FC ≥ -1.0; p < 0.05) (Table 2) [74–76]. Notably, the levels of docosahexaenoic acid (DHA), a neuroprotective agent that helps minimise the risk of lifelong neurological impairment resulting from brain injury [42], GABA, a primary inhibitory neurotransmitter, and L-tryptophan (Trp), an essential precursor of the monoaminergic neurotransmitter serotonin (5-hydroxytryptamine, 5-HT), were depleted following ICV polymyxin B treatment (log2FC ≥ -4.0; p < 0.05) (Table 2). Four biomarkers involved in energy production in the brain, namely creatine (a mediator of brain ATP production), acetyl carnitine (ALCAR; an agent enhances energy status, reduces oxidative stress), ATP and L-fructose 1,6-biphosphate (a crucial mediator in astrocytes glucose-neogenesis) were also significantly decreased in response to ICV polymyxin B treatment (log2FC ≥ -2.0; p < 0.05) (Table 2) [77–80].

2.12. Pathway enrichment analysis for significantly perturbed metabolites of the left cerebral cortex following ICV polymyxin B treatment

As ICV polymyxin B induced extensive perturbations in the metabolome of the left cortex largely at 4 h, with negligible effects
seen at 1 h, we only mapped the significantly impacted metabolic features of the former time point (Supplementary Fig. S4A and B; Supplementary Fig. S5B). Mapping of the significantly perturbed metabolites of the left cerebral cortex at 4 h into biochemical pathways revealed that the biosynthesis of unsaturated fatty acids (4 intermediates), linoleic acid (3 intermediates), sphingolipid (7 intermediates) and arachidonic acid metabolism (2 intermediates), were amongst the most significantly impacted pathways (Supplementary Fig. S5B).

2.13. Polymyxin B distribution in different regions of the rat brain following ICV administration

Localization of polymyxin B was determined by measuring polymyxin B concentrations in the four main parts of the brain (cerebellum, brain stem, cortex, and hippocampus) at 1 h using LC-MS. As polymyxin B was delivered directly to the right lateral ventricle according to our previously published methods [34], accordingly polymyxin B was mainly located in the right and left cortices followed by the right hippocampus and to a lesser extend in the brain stem (Supplementary Table S2).

3. Conclusions

This is the first metabolomics study to investigate the mechanisms underlying the neurotoxicity of polymyxin B following administration directly into the brain via the ICV route. ICV polymyxin B treatment of rats significantly perturbed neuronal lipid homeostasis, brain antioxidant status and mitochondrial function. Furthermore, the levels of key metabolites that act as neuroprotectants and neuromodulators (e.g., NAG and glutamate) in response to cerebral ischemia, oxidative stress, and acute brain injury were significantly perturbed. Given that the direct administration of polymyxin B into the brain is the most efficient route of delivery for the treatment of CNS bacterial infections, a proper balance between antibacterial efficacy and neurotoxicity is inevitably the primary clinical goal of optimising the use of these important last line antibiotics. Our study provides a fundamental understanding of the metabolic neurotoxic mechanisms associated with ICV polymyxin B that will help facilitate future PK/PD programs aimed at optimising the clinical use of CNS polymyxins.

4. Materials and methods

4.1. Ethics approval

Experimental protocols were approved by the Animal Ethics Committee of the University of Melbourne (IACUC#1914890). All animal procedures conform to the NIH guidelines on the protection of animals and are in accordance with the Australian code for the care and use of animals for scientific purposes.

4.2. Reagents

Polymyxin B (Beta Pharma, China # 20120204) solutions were freshly prepared in 0.9 % NaCl and vortexed until fully dissolved prior to each experiment. Cyclophosphamide powder (Clifford Hallam Healthcare, Victoria, Australia) was reconstituted in 0.9 % NaCl according to the manufacturer’s instructions to obtain a working solution of 20 mg/mL and was stored at 4 °C thereafter for a maximum of one week.

4.3. Animal experimental design

Six male, 9-week-old Sprague-Dawley rats bred in house or acquired from the Animal Research Centre (Perth, Australia) were used for the experiments. The animals were housed in standard open top cages at 21 °C and maintained on a 12 h light/dark cycle (lights on at 0700–1900), with ad libitum access to chow and water. Animals were group housed before surgery and single housed post-surgery, with the standard lid being replaced by a lid with a raised food hopper to avoid their head mounts being caught in the wire lid. On day 0, cerebroventricular cannulation of the right lateral ventricle was performed by surgically implanting a guide cannula. On Day 1, following 24 h of recovery the rats were ready for ICV injection of either polymyxin B or 0.9 % NaCl [34].

4.4. Drug administration

Polymyxin B 0.75 mg/kg or 0.9 % NaCl was administered into the right lateral ventricle using the standard dosing setup consisting of an internal cannula fed down the implanted guide cannula attached into a microsyringe (Hamilton, Nevada, USA) by PE-50 tubing [34]. The appropriate dosing volume (between ~ 4–6 μL) was calculated based on the rats’ body weight. The internal cannula was held in place for 10 sec following administration of the injection volume to avoid any backflow out of the guide cannula.

4.5. Sample collection

Rats were anaesthetized with 5 % isoflurane (in oxygen) in an induction chamber for 5 min until the proper plane of anaesthesia i.e., pedal reflex was lost. Animals were euthanized ~ 1 and 4 h post injection by cardiac puncture following the removal of the brain. The brain stem and cerebellum were collected and snap-frozen on dry ice. The remaining brain tissue was dissected on an ice-cold matrix. The hippocampus and cortex were separated for each hemisphere and snap-frozen on dry ice, and all remaining brain parts were labelled as ‘remainder’. All samples were stored at ~ 80 °C until processing.

4.6. Sample preparation for polymyxin B distribution experiment

The LC-MS samples were prepared for measurement of polymyxin B concentrations in different brain regions as described in the Supplementary information.

4.7. Sample preparation and metabolite extraction

The frozen tissue was pulverised using steel tissue pulveriser. Six frozen tissue samples were dipped in a cold pulveriser cooled in a bath of liquid nitrogen for ~ 1 min. The tissue samples were then crushed by hammering in a stainless steel well (one by one) and the powdered tissue was immediately scraped and transferred into an Eppendorf tube. The tissue was weighed (~35 mg) and the extraction solvent (1:20 solvent/tissue ratio) 25 ml of MeOH/H2O (v/v 4:1) spiked with 100 μM glutamic acid (25 μL of 100 mM solution) and 70 μM of glutamate (17.5 μL of 100 mM solution) containing 1 μM each the internal standards (3-[3-chola midopropyl]-dimethylammonio]-1-propanesulfonate [CHAPS], N-cyclohexyl-3-aminopropanesulfonic acid [CAPS], piperazine-N,N’-bis(2-ethanesulfonic acid) [PIPER], and Tris) were added. Subsequently, the samples were sonicated for 30 min in an ice bath followed by vortexing at 4 °C for 15 min. Finally, the samples were spun at 4 °C and 14,000 x g for 10 min. Then 100 μL of the supernatant was transferred to LCMS vials. An equal volume of supernatant from each of the six samples was combined to make a
quality control (QC) sample. The samples were stored at −80 °C until the analysis.

4.8. Metabolomics data processing and statistical analyses

IDEOM, a graphical interface in Microsoft Excel was used for processing raw LC-MS data files (.mzXML) using XCMS and mzmatch.R tools for automatic noise filtering and identification of metabolite features [81]. Firstly, the raw peaks extracted by XCMS (peakML files) [82], were filtered and aligned using Mzmatch.R tool with a minimum detectable intensity of 100,000, an RSD of < 0.5 (reproducibility), and a peak shape (codawd) of > 0.8. Mzmatch was used for retrieving and annotating the missing and related peaks respectively. Default IDEOM parameters were utilized to eliminate common sources of noise such as chromato-metric peak shoulders, contaminant signals, irreproducible peaks, in addition to the background noise. A negative or positive electrospray ionization (ESI) mode was employed to correct the loss or gain of a proton, respectively, followed by a data-dependent polynomial mass recalibration step (2 ppm) for the putative metabolites. Putative metabolites were identified and annotated by matching the accurate mass and retention times of the obtained peaks to the corresponding metabolite in the incorporated database, including the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, and LIPIDMAPS databases, using preference for central metabolites profiled in BioCyc. The detected metabolites were verified using the retention time of the authentic standards. For the identification of metabolites showing differential response, they were subjected to statistical analysis using MetaboAnalyst 5.0, a free online tool. Briefly, the extracted table of putative metabolites [median RSD of ≤ 0.2 (%) within the QC group; confidence level of ≥ 5] was uploaded to MetaboAnalyst. Data with > 25 % missing values were deleted, and the remaining missing values were replaced by 1/5 the minimum positive value of the corresponding variable [83]. Data filtering was performed using the robust estimate, the interquartile range (IQR), followed by normalization, log transformation, and autoscaling. The significantly perturbed metabolites were determined by comparing the treatment and untreated control = groups using T-test (p-value < 0.05; log2FC > 0.59). The pathway mapping was performed by uploading the KEGG compound numbers of significantly changed metabolites into MetaboAnalyst 5.0 and the selected pathway library was Rattus norvegicus (rat).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.10.041.

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