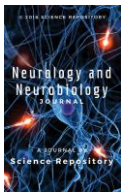

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Research Article

# Metabolomics of Rat Brain After Treatment with Phenelzine: High-Resolution Mass Spectrometric Demonstration of Increased Brain Levels of N-Acetyl Amino Acids

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ABSTRACT

**Background:** Phenelzine (PLZ) is a non-specific monoamine oxidase inhibitor that has demonstrated clinical efficacy in patients with treatment resistant depression. The mechanism of action with regard to this efficacy is complicated in that its metabolite, β-phenylethylidenehydrazine (PEH), is an inhibitor of amino acid transaminases resulting in dramatic brain elevations of GABA, alanine, ornithine and tyrosine. The full neurochemical profile of PLZ and PEH remain to be explored.

**Objective:** To undertake a non-targeted metabolomics study of phenelzine on rat brain neurochemistry.

**Methods:** We undertook a high-resolution mass spectrometric metabolomics analysis of rat cortical brain 1 and 12 hours after intraperitoneal dosing with PLZ or PEH. Tandem mass spectrometry was utilized to obtain relative quantitation data.

**Results:** N-acetyl amino acids were found to be elevated in cortical brain tissue following either PLZ or PEH treatments.

**Conclusions:** Our data indicate PLZ treatment significantly augments brain levels of N-acetyl amino acids and that this may involve inhibition of deacylases by PEH and/or induction of N-amino acid acetyltransferases.



## Introduction

Phenelzine is a nonspecific monoamine oxidase (MAO) inhibitor that has been utilized in patients with treatment resistant depression [1-4]. Phenelzine is a potent inhibitor of both MAO-A and MAO-B which increases brain monoamine levels. In contrast, its metabolite, β-phenylethylidenehydrazine (PEH), is a weak MAO inhibitor but is a potent transaminase inhibitor that significantly elevates brain levels of GABA and alanine, tyrosine, and ornithine [5-8]. PLZ and PEH also are both carbonyl scavenging agents that reduce reactive aldehyde toxicity *in vivo* and *in vitro*. The aldehyde scavenging actions of PLZ and PEH have been hypothesized to be responsible for their neuroprotective actions in animal models of ischemia-reperfusion injury, traumatic brain injury, spinal cord injury, experimental autoimmune encephalitis, and *in vitro* aldehyde toxicity [9-14]. These data indicate that PLZ and PEH have very complex pharmacodynamic effects. To further investigate the biochemical effects of these compounds, we undertook a non-targeted metabolomics study of PLZ and PEH on the rat brain metabolome. This study revealed that PLZ and PEH significantly augment brain levels of free N-acetyl amino acids.

## Methods

### I Rat Brain Samples

Male Sprague Dawley rats were dosed intraperitonially with 30 mg/kg of PLZ or PH, and the brains harvested after decapitation at 1 and 12 hours. The brains were frozen immediately in isopentane on solid carbon dioxide and then removed to containers stored at -80ºC until the frontal cortex was dissected out for metabolomics analysis. All procedures

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involving animals were approved by the University of Alberta Biosciences Animal Care and Use Committee (AUP00000216) and were in accordance with the guidelines of the Canadian Council on Animal Care.

### II Sample Processing

40 to 60 mg of cortical tissue were sonicated in 1 mL of ice-cold acetonitrile:methanol:formic acid (800:200:2.5) containing the stable isotope internal standards [$^2H_3$]N-acetyl-methionine, [$^2H_5$]N-acetyl-glutamate, and bromocriptine [15]. After centrifugation at 30,000 x g and 4ºC for 30 min, 750 µL of the clear supernatant was dried by centrifugal vacuum evaporation. The samples were next dissolved in acetonitrile:methanol (1:1) for flow infusion analyses.

### III High-Resolution Mass Spectrometric Analyses

Samples underwent flow infusion analyses at a flow rate of 12 µL per min. and were analysed via high-resolution mass spectrometry (HR-MS) utilizing a Q-Exactive benchtop orbitrap (Thermo Fisher) with a resolution of 140,000 and less than 1 ppm mass error. Negative ion electrospray ionization (NESI) with a sheath gas of 12, a spray voltage of 3.7 kV, and a capillary temperature of 321ºC was used. For the pilot metabolomics analysis, the scan was from 60 to 900 amu. The data were analysed via an in-house Excel (Microsoft) spreadsheet with over 1200 metabolites of interest.

To obtain relative quantitative data of N-acetyl amino acids, $MS^2$ studies utilized a window of 0.4 amu for the precursor ion and the product ions were acquired at high resolution (< 1 ppm mass error). For $MS^2$ studies (Table 1) the neutral collision energy (NCE) was optimized between 20 and 30 eV.

**Table 1:** $MS^2$ analyses of N-acetyl amino acids.

| N-Acetyl Amino Acid | $[M-H]^-$ | $MS^2$ Product | Product Anion |
|---|---|---|---|
| N-Acetyl Glycine | 116.0352 | Gly | 74.0247 |
| N-Acetyl Proline | 156.0665 | Pro | 114.0717 |
| N-Acetyl Valine | 158.0822 | Val | 116.0717 |
| N-Acetyl Threonine | 160.0615 | Thr | 118.0509 |
| N-Acetyl Hydroxyproline | 172.0615 | Hydroxyproline | 130.0509 |
| N-Acetyl Leucine | 172.0979 | Leucine | 130.0873 |
| N-Acetyl Glutamine | 187.0724 | Glutamine | 145.0618 |
| N-Acetyl Glutamate | 188.0564 | Glutamate-$CO_2$ | 102.0560 |
| N-Acetyl [$^2H_5$]Glutamate | 193.0731 | [$^2H_5$]Glutamate – $CO_2$ | 107.0873 |
| N-Acetyl-Methionine | 190.0547 | Methionine | 148.0437 |
| N-Acetyl [$^2H_3$]Methionine | 193.0731 | [$^2H_3$]Methionine | 151.0626 |

### IV Data Presentation

Data are presented as relative (R) N-acetyl amino acid levels (i.e. the ratio of the signal intensity of the endogenous N-acetyl amino acid to the signal intensity of an appropriate stable isotope internal standard), corrected for protein, ± SD (N=5).

### Results

The most marked observation from our preliminary non-targeted metabolomics analysis was increased brain levels of N-acetyl amino acids with PLZ and PEH dosing. Since this was a flow infusion analysis, there were a number of potential isobars with the exact mass of each N-acetyl amino acid. Therefore, to obtain relative quantitation data we performed $MS^2$ analyses. This approach clearly demonstrated increased levels of an array of brain N-acetyl amino acids (Figure 1). It is interesting to note that N-acetylaspartate levels, which are in millimolar (mM) concentrations in the brain, were unaffected by the drug treatments.

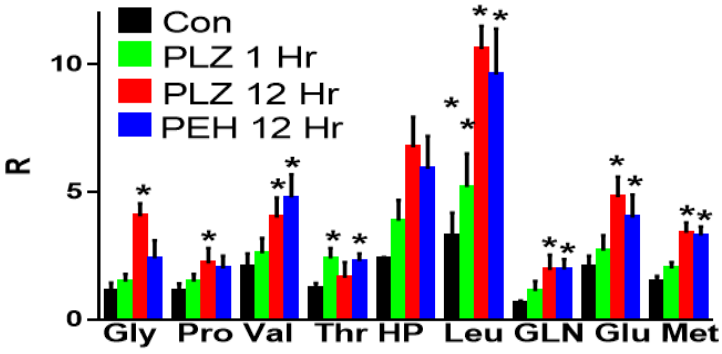


**Figure 1:** Relative levels (R) of N-acetyl amino acids in rat cortical brain tissue at 1 and 12 hours after treatment with phenelzine (PLZ, 30 mg/kg, i.p.) or β-phenylethylidenehydrazine (PEH). Gly: glycine; Pro: proline; Val: valine; Thr: threonine; Hyp: hydroxyproline; Leu: leucine/isoleucine; Gln: glutamine; Glu: glutamate; Met: methionine. Mean ± SEM (N=5); *, $p < 0.05$.

## Discussion

While our knowledge base regarding post-translational processing of proteins, via N-acetylation of serine, alanine, glycine, methionine, threonine, valine, and aspartate (EC 2.3.1.254-258), or lysine (EC 2.3.1.32) has grown significantly, our understanding of the roles of free N-acetyl amino acids is much more limited [16, 17]. Protein bound N-acetyl amino acids are released by acylaminoacyl peptidase (EC 3.4.19.1); however, free N-acetyl amino acids are also synthesized by a number of N-acetyl transferases. The most studied free N-acetyl amino acid is N-acetylaspartate, since it is present in millimolar concentrations in the brain [18, 19]. N-Acetylaspartate is synthesized by a specific acetylase NAT8L (EC 2.3.1.17) [20, 21]. Whereas N-acetyl glutamate and N-acetylmethionine are both synthesized via amino acid N-acetyltransferase (EC 2.3.1.1) [22, 23]. Interestingly, methamphetamine, a monoamine releaser, has been shown to induce NAT8, the aforementioned synthetic enzyme involved in the biosynthesis of N-acetylaspartate [24, 25]. PLZ, which augments monoamine levels, may therefore also induce NAT8L and augment N-acetylamino acids. However, PEH which is a weak MAO inhibitor, could not be acting via this mechanism.

Alternatively, inhibition of catabolism might be involved in the augmentation of N-acetyl amino acids, similar to the augmentation of amino acids via inhibition of transaminases by PEH [7]. The potential enzyme targets include aliphatic aminoacylase (EC 3.5.1.14; ACY1) [26, 27], aspartoacylase (EC 3.5.1.15; ACY2), and N-amino aromatic amino acid amidohydrolase (EC 3.5.1.114; ACY3) [28, 29]. In this regard, inhibition of ACY3 provides neuroprotection from aldehyde toxicity, suggesting that in addition to carbonyl scavenging, augmentation of N-acetyl amino acids may contribute to the neuroprotection provided by PLZ in models of neural trauma [9-14, 29].

The functional roles of free N-acetyl amino acids remain to be more fully elucidated but decreased plasma levels of N-acetylmethionine have been monitored in cystic fibrosis patients and childhood obesity, and decreased plasma levels of N-acetylglycine in obesity [30-33]. Cadmium, which is an ACY1 inhibitor, elevates urinary levels of N-acetylglutamate, N-acetylglutamine, and N-acetylphenylalanine, suggesting a rapid turnover rate for these N-acetyl amino acids *in vivo* [34, 35]. Similarly, precursor labeling studies have defined the rapid dynamics of N-acetylmethionine synthesis in human oligodendrocyte cultures [22].

A detailed analysis of N-acetyl amino acids in dogs with gallbladder mucocele formation found decreased blood levels of N-acetylated alanine, glycine, glutamate, isoleucine, leucine, methionine, serine, and threonine [35]. In contrast, the bile in these dogs was characterized by increased levels of N-acetylated glutamate, histidine, isoleucine, leucine, lysine, threonine, tryptophan, tyrosine, and valine. These data suggest that N-acetyl amino acids play a complex metabolic function in the gallbladder.

With regard to brain function, free N-acetylmethionine, N-acetylglutamine, N-acetylglutamate, N-acetylasparagine, and N-acetylalanine have all been monitored in the human brain [23, 36-40]. N-acetylglutamate is a critical modulator of the urea cycle, while N-acetylleucine modulates the neuronal activity of vestibulocerebellar and posterolateral thalamic circuits involved in vestibular function, while N-acetylglutamine appears to be involved in the sleep-wake cycle [40-42]. Clearly, we currently have limited knowledge of the roles of N-acetyl amino acids in brain function.

## Conflicts of Interest

None.

## Funding

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