GC–MS Metabolomic Analysis Reveals Significant Alterations in Cerebellar Metabolic Physiology in a Mouse Model of Adult Onset Hypothyroidism

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Abstract: Although adult-onset hypothyroidism (AOH) has been connected to neural activity alterations, including movement, behavioral, and mental dysfunctions, the underlying changes in brain metabolic physiology have not been investigated in a systemic and systematic way. The current knowledge remains fragmented, referring to different experimental setups and recovered from various brain regions. In this study, we developed and applied a gas chromatography—mass spectrometry (GC-MS) metabolomics protocol to obtain a holistic view of the cerebellar metabolic physiology in a Balb/cJ mouse model of prolonged adult-onset hypothyroidism induced by a 64-day treatment with 1% potassium perchlorate in the drinking water of the animals. The high-throughput analysis enabled the correlation between multiple parallel-occurring metabolic phenomena; some have been previously related to AOH, while others implicated new pathways, designating new directions for further research.

Introduction

Although the adult-onset hypothyroidism (AOH) is accompanied by movement1 and psychobehavioral/mental dysfunctions,2,3 the adult mammalian brain has been considered metabolically nonresponsive to thyroid hormones (TH). This perception was initiated from an early study of Fazekas et al.,4 reinforced later by a tightly regulated T3 maintenance mechanism in the adult hypothyroid brain [for review, see ref 5]. The above-mentioned neurological dysfunctions are thought to be the result of AOH effects on peripheral organs, mainly the liver and the heart.1 However, the central and direct role of TH on brain function, in general, and brain metabolism, in particular, has been supported by several studies [for review see refs 1, 6] establishing thus the adult mammalian brain as a TH target tissue of particular interest for biomolecular research.

The adult mammalian brain has a large number of high-affinity T3 nuclear receptors (TRs),7 which act as transcription factors. Even though the number of TRs is affected by changes in the thyroidal state,8 no significant transcriptional alterations due to AOH have been observed in rodent studies.9 A mechanism explaining the T3-independent transcriptional regulation of the adult brain has been proposed.10 Furthermore, minimal changes in targeted protein concentrations due to AOH have been detected.1,8 However, TH effects on brain signaling [for review see refs 6, 11] and metabolic processes have been revealed, indicating that TH cellular actions could also be initiated at the plasma membrane, in the cytoplasm and at the mitochondrion.12 These designate the need to further investigate in a systemic and systematic way the regulation of brain signaling and metabolism in relation to TH. For the effect of AOH on brain metabolism, in particular, the current knowledge remains fragmented, referring to different experimental setups and recovered from various brain regions.13–21 Earlier studies support that the AOH effects on the metabolic function of the brain are region-specific.22 Thus, obtaining a holistic view of metabolism in particular brain regions under AOH is expected.
to significantly enhance the current knowledge about the disease. High-throughput metabolic studies became possible with the development of metabolomic technologies in the systems biology era.

In this biological and technological context, we developed and applied a gas chromatography–mass spectrometry (GC–MS) metabolomics protocol to study the cerebellar metabolic physiology of the Balb/c mouse under prolonged AOH induced by a 64-day treatment with 1% potassium perchlorate in the drinking water of the animals. Mouse models can provide significant data about the disease, which could be appropriately extrapolated to human. Metabolomics refers to the high-throughput quantification of the (relative) concentration of the free small metabolites and enables the monitoring of a metabolic fingerprint, which complements the transcriptomic and proteomic profiles of a biological system. Metabolomics can be easily used to monitor transient metabolic conditions without requiring extensive knowledge of the structure and regulation of the investigated metabolic networks. GC–MS metabolomics focuses on primary metabolism. These characteristics are advantageous for the analysis of the AOH effects on brain metabolism, considering the multiplicity of the already identified molecular and biochemical events that accompany AOH in various levels of brain function, combined with the anatomical, morphological and phenotypic complexity of this organ. This study highlights the significance of the systemic analysis of the AOH effects on brain metabolic physiology. It also contributes to the technology development of brain tissue metabolomics.

The selection of cerebellum as the target brain region of this study was based primarily on the direct connection of AOH with phenotypic alterations of the cerebellum core functions, including ataxia and lack of motor coordination. These symptoms have been mainly attributed to morphological alterations, changes in myelin sheath turnover, and modification in several neurotransmitter systems. In this paper, it will be shown that significant metabolic alterations also occur, which could further support the phenotypic manifestations of the disease into movement dysfunctions. Moreover, considering that the role of the cerebellum to neurocognition may be more substantial than originally believed, the implications of the observed cerebellar metabolic alterations on the overall brain physiology could be even broader.

Materials and Methods

Experimental Design. All 11 male Balb/cJ mice of the study were fed with laboratory chow (i.e., mouse rich complex food in the form of pellets #BPI-302 from Viozois S.A. (aEL 3300001), Ioannina, Greece) and water *ad libitum* until the 60th day of their life (P60). On P60, the mice were randomly separated into two groups of five (5) and six (6), respectively, and each group was housed in the same cage until sacrifice. The 5 mice of the first group (it will be referred to as “euthyroid”) were kept under the same diet for the rest of the experiment. On P60 and until the day of sacrifice (P124), 1% KClO₄ was added to the drinking water of the second group (it will be referred to as “hypothyroid”). On P124, all mice were sacrificed and their cerebellum was isolated for analysis, as described in the next section. GC–MS was used to acquire the P124 polar metabolic profiles of the cerebellum in both groups.

The particular AOH mouse model has been optimized in the Human and Animal Physiology Laboratory of the Biology Department, University of Patras, Greece. KClO₄ has been one of the agents used for the induction of hypothyroidism in animals (for a thorough discussion, see ref 32) and the only known not to affect enzymatic activities. The 2 month treatment duration has been matched to prolonged AOH based on relevant experiments for the optimization of the animal model. These experiments (number of mice: 8–10 per experiment, *p* < 0.05) indicated that, after 8 weeks of the KClO₄ treatment, the T₃ and T₄ levels in the blood plasma of the euthyroid mice are, respectively, 1.6 ± 0.27 and 58 ± 3.0 ng/mL while those of the hypothyroid mice, 0.25 ± 0.03 and 25 ± 1.2 ng/mL. In the same set of animals, the time profile of the mean body weight (BW) in the euthyroid and hypothyroid group is shown in Supplementary File 1, confirming the connection between the induction of AOH and the reduced rate of the body weight (BW) increase in this AOH model. At the end of the 8-week treatment, the mean BW of the hypothyroid was 68.8% of the mean BW of the euthyroid group.

Animal Housing and Treatment. The animals belonged to the Balb/cJ mouse colony of the Human and Animal Physiology Laboratory of the Biology Department, University of Patras, Greece. They were housed and bred in the Animal House Facility of the University General Hospital of Rio, Patras, Greece, according to the standards of the international statutes on animal housing and handling (86/609/EEC). All animals were exposed to regular light–dark cycle (i.e., light period: 7 a.m. to 7 p.m.; dark period: 7 p.m. to 7 a.m.) at 22 ± 1°C throughout the duration of the experiment. KClO₄ was procured from Merck-Hellas. Starting from P60 and until sacrifice, all 11 animals were weighed in approximately 3-day intervals to monitor and evaluate the progress and effectiveness of the KClO₄-induced AOH through the reduced rate of BW increase. On P124, between 9:00 a.m. and 11:00 a.m., the animals were sacrificed by decapitation under light ether anesthesia, according to the international statues of animal handling for pain minimization (86/609/EEC). After sacrifice, whole brains were removed on a sterile ice-cold glass plate, and the cerebellum of each animal was immediately isolated. Subsequently, each tissue sample was rapidly weighed (Table 1), frozen in liquid nitrogen, and stored at −80°C until further analysis. The entire process from the removal of the brain to the quenching of the various brain regions lasted less than 1 min.

Metabolic Profiling Analysis. The polar metabolite extracts of the cerebellum from each animal were obtained based on a methanol/water extraction protocol adapted from respective protocols of our group. Specifically, 22 mL of HPLC-grade methanol (SDS SA, Puype, France), 0.1 mg of each of the internal standards, ribitol (Alfa Aesar, U.K.), and [U-¹³C] glucose (Cambridge Isotope Laboratories, MA) were added per 1 g of frozen tissue. On the basis of previous measurements in Klapa’s laboratory (data not shown), endogenous ribitol was detected neither in eu- nor in hypothyroid mouse cerebellum. The dried polar extracts were derivatized into their methoxime trimethylsilyl- (or (MeOx)TMS-) derivatives through reaction with 150 µL of 20 mg/mL methoxyamine hydrochloride solution in pyridine for 90 min, followed by reaction with 300 µL of N-methyltrimethylsilyl-trifluoroacetamide (MSTFA) for at least 6 h at room temperature, according to the derivatization strategy described previously. All reagents used in the derivatization step were procured from Alfa Aesar, U.K. The metabolic profiles of the derivative samples were acquired using the Saturn 2200 gas chromatograph–ion trap mass spectrometer (Varian, Inc., CA) at 1:25 split ratio (GC column: Phenomenex #7HG-G004-11-B). The peak identification and
quantification was carried out as described in Kanani and Klapa. The raw metabolic data set is provided in Supplementary File 2; it comprises 95 peaks, each of which was detected in at least one of the acquired metabolic profiles and corresponds to a compound of known chemical category according to the category definition described earlier. Euthyroid animal 4 (4E) was excluded from further analysis, because its acquired polar metabolic profiles (at four different derivatization times) were suspected to be subject of experimental biases that affect differently each amine-group containing metabolite in the profile and cannot be corrected either through the use of the internal standard or the correction strategy described in Kanani and Klapa. Specifically, peaks that were expected to be among the highest (e.g., phosphoric acid, one of the aspartate derivatives) were measured with very small peak areas throughout all injections of this sample, without, however, a corresponding decrease in other peak areas of the profile. Of note, in all acquired chromatograms, endogenous glucose appeared only in the form of glucopyranose (in other brain regions that have been studied as well; data not shown). This observation requires further exploration, because typically, incomplete methoximation, which can give rise to glucopyranose peaks, manifests in both glucose and (smaller) glucopyranose peaks being present. The relative areas of all detected peaks (RPAs, Relative Peak Areas) were estimated from their normalization with the 217 glucose and (smaller) glucopyranose peaks being present. Of note, in all acquired chromatograms, endogenous glucose appeared only in the form of glucopyranose (in other brain regions that have been studied as well; data not shown). This observation requires further exploration, because typically, incomplete methoximation, which can give rise to glucopyranose peaks, manifests in both glucose and (smaller) glucopyranose peaks being present. The relative areas of all detected peaks (RPAs, Relative Peak Areas) were estimated from their normalization with the 217 glucose and (smaller) glucopyranose peaks being present.

The relative areas of all detected peaks (RPAs, Relative Peak Areas) were estimated from their normalization with the 217 marker ion peak area of the internal standard ribitol. The recently developed data validation, normalization, and correction methodology was applied to account for the derivatization biases that are primarily due to the formation of multiple derivatives from the amine-group containing metabolites. Specifically, we first verified same GC–MS operational conditions during the acquisition of all metabolic profiles according to the criterion in Kanani and Klapa. Then, the derivative peak areas that corresponded to the same amine-group containing metabolites were combined into one cumulative (effective) peak area, using the weight coefficients that were estimated based on the profiles of euthyroid 1 (1E) (for serine, glycine, leucine, isoleucine, and tyrosine) and euthyroid 2 (2E) (for aspartate, β-alanine, glutamate, and norleucine) samples. From the detected amine-group containing metabolites, valine, ethanolamine, lysine, alanine, and threonine were filtered out of further analysis, because their available measurements did not allow for all positive weight coefficients to be estimated. The amine-group containing metabolites, for which only one derivative was observed in the particular derivatization range (i.e., proline, uracil, GABA, cysteine, methionine, phenylalanine, and histidine), but for which more than one derivative are known, were included; most often they are filtered out at a later step (in this case this happened for proline, cysteine, methionine, phenylalanine, and histidine), because of high coefficient of variation between injections (see below). Further, (a) the smallest of the two MeOx peaks of the known ketone-group containing metabolites [see explanation in ref 34], (b) the peaks corresponding to unknown amine-group containing metabolites [see explanation in ref 34], (c) the peaks (i) that were identified as derivatization artifacts, or (ii) with significant carryover, or (iii) that were inconsistently detected among samples and/or injections of the same sample, or (iv) with significant variation between injections, were filtered out of the analysis. The profiles of injections 1E-Inj3 and 2E-Inj3 (see Supplementary File 2) were filtered out of the analysis as increasing the variance between the injections of the corresponding samples. After this normalization and filtering step, the final RPA profiles that were used in data analysis included 46 metabolites (shown in light green background in Supplementary File 2). Any missing RPAs were imputed using the k-nearest neighbor’s algorithm, as is implemented in the TM4 MeV (v. 4.4.1) software.

### Table 1. Tissue Sample and Dry Polar Metabolite Extract Weights

<table>
<thead>
<tr>
<th>animal no.</th>
<th>tissue sample</th>
<th>tissue sample weight (TSW) (mg)</th>
<th>dry polar metabolite extract weight (DPMEW) (mg)</th>
<th>DPMEW/TSW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>1E</td>
<td>1E-Ce</td>
<td>57.3</td>
<td>4.9</td>
</tr>
<tr>
<td>n = 5b</td>
<td>2E</td>
<td>2E-Ce</td>
<td>59.8</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>3E</td>
<td>3E-Ce</td>
<td>68.3</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>4E</td>
<td>4E-Ce</td>
<td>64.1</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>5E</td>
<td>5E-Ce</td>
<td>57.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>61.4 ± 4.7</td>
<td>5.5 ± 0.75</td>
</tr>
</tbody>
</table>

Hypothyroid

<table>
<thead>
<tr>
<th>animal no.</th>
<th>tissue sample</th>
<th>tissue sample weight (TSW) (mg)</th>
<th>dry polar metabolite extract weight (DPMEW) (mg)</th>
<th>DPMEW/TSW (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>1H-Ce</td>
<td>67.7</td>
<td>5.9</td>
<td>8.7</td>
</tr>
<tr>
<td>n = 6</td>
<td>2H</td>
<td>2H-Ce</td>
<td>60.5</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>3H</td>
<td>3H-Ce</td>
<td>64.2</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>4H</td>
<td>4H-Ce</td>
<td>57.6</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>5H</td>
<td>5H-Ce</td>
<td>65.3</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>6H</td>
<td>6H-Ce</td>
<td>62.0</td>
<td>6.4</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td>62.9 ± 3.6</td>
<td>7.0 ± 1.4</td>
</tr>
</tbody>
</table>

*Ce, SD stand for Cerebellum and standard deviation, respectively. b The polar metabolic profiles of the tissue samples from the animals indicated in bold were used in the present analysis. The reasons for the exclusion of the 4E-Ce polar metabolic profile are stated in Materials and Methods.
cerebellar samples was obtained by positioning the metabolites identified as significant in the appropriately color-coded metabolic network. The reconstruction of the metabolic network was mainly based on information from Siegel et al.38 and the metabolic databases KEGG39 and EXPASY.40 Any other reference used for specific reactions is cited in the next sections.

Results

Effect of Prolonged Treatment with KClO₄ on the BW Time Profiles. The BW time profiles for all animals from P60 to P124 are shown in Figure 1. On P60, there was no significant difference in the mean BW between the two animal groups. For the duration of the treatment, the BWs of the euthyroid group kept increasing. At the end of the treatment period, the hypothyroid group mean BW was 63% of the euthyroid mean. Animals 1E–5E, 1H–6H: Euthyroid and Hypothyroid, respectively, male Balb/cJ mice.

![Figure 1. The BW time profiles for the treatment period. A BW decrease was observed in the hypothyroid group after ~40 days of treatment; on the contrary, the BWs of the euthyroid group kept increasing. At the end of the treatment period, the hypothyroid group mean BW was 63% of the euthyroid mean. Animals 1E–5E, 1H–6H: Euthyroid and Hypothyroid, respectively, male Balb/cJ mice.](image)

From Figure 1, it could also be observed that (a) 5E was the animal with the largest increase in its BW within the euthyroid group, (b) 1H showed fluctuations in its BW after the 40th day of treatment; 1H was the heaviest of all 11 animals at the beginning of treatment on P60, and (c) 5H–6H were the lightest of all animals on P60 and remained so throughout the treatment period. No difference was observed in the mean cerebellum weight between the euthyroid and the hypothyroid group on P124 (Table 1). Moreover, the mean concentration of the extracted polar metabolites in the cerebellum tissue was similar between the two groups (Table 1).

The Metabolites Whose Concentration Changes Significantly between Hypothyroid and Euthyroid Cerebella. Significance analysis between the euthyroid and the hypothyroid polar metabolic profiles for zero (0) False Discovery Rate (FDR)-median identified, respectively, 2 (1 of unknown identity) and 17 (2 of unknown identity) positively and negatively significant metabolites, which are shown in Table 2; the 2 positively and the first 12 negatively significant correspond also to the stricter criterion of zero (0) FDR-90-th percentile. From the SAM curve in Figure 2, it becomes apparent that the first 4 negatively significant metabolites, i.e. aspartate, scyllo-inositol, glucose (in the form of glucopyranose), and fumarate, which correspond to the 4 outer left green points on the graph, are the most discriminatory between the two groups. At the threshold of significance, which reveals only these four metabolites as negatively significant, no metabolite is identified as positively significant (see first 4 rows of Table 2). The HCL analysis (Figure 3A) and the 3-D PCA graph (Figure 3B) of the RPA profiles of the significant metabolites for all animals validate that the hypothyroid could indeed be differentiated from the euthyroid mice based on the concentration profile of these metabolites.

Supplementary File 3 discusses the significance analysis results when the 5E and 1H polar metabolic profiles are excluded from the analysis. These two profiles were observed as increasing the biological variance of the euthyroid and hypothyroid groups, respectively (see details in Supplementary File 4). Very few differences and only among the lower level of significance metabolites were identified between the two analyses (see Supplementary File 4). Actually, the additional known metabolites that were identified as negatively significant, i.e., glutamate, monomethylphosphate, and galactose complement the map of the significant alterations in the

### Table 2. The Positively and Negatively Significant Metabolites in the Hypothyroid with Respect to the Euthyroid Cerebellar Samples, Based on Unpaired SAM Analysis for the Designated Significance Threshold Value

<table>
<thead>
<tr>
<th>Order of Significance</th>
<th>Negatively Significant Metabolite no.</th>
<th>Negatively Significant Metabolite name</th>
<th>Positively Significant Metabolite no.</th>
<th>Positively Significant Metabolite name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>aspartate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>chloro- (or scyllo)-inositol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>glucose (in the form of glucopyranose)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>fumarate</td>
<td>1. unknown_114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>norleucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>GABA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>urea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>uracil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>unknown_107</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>leucine</td>
<td>2. threonate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>β-alanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Benzoate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>unknown_121</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>oxalate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>2-hydroxyglutarate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>myo-inositol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>2-hydroxybutanoic acid</td>
<td></td>
<td></td>
<td></td>
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</table>

*See SAM diagram in Figure 2. Both significance threshold values, d1 and d2, correspond to zero False Discovery Rate (FDR)-median; d1 also corresponds to zero FDR-90-th percentile (i.e. stricter SAM). SAM using d2 corresponds to FDR-90-th percentile equal to 0.65 (3.43%) false positives. The metabolites are shown in decreasing order of significance. In the SAM graph (Figure 2), the order of significance decreases from the outer to the inner metabolite points.
cerebellar metabolic physiology due to AOH that can be reconstructed based on the 19 significant metabolites of Table 2 (see next section).

**Map of Differences in Metabolic Physiology between Hypothyroid and Euthyroid Cerebellum.** Figure 4 provides a map of the significant changes in the metabolic physiology of the adult hypothyroid compared to the euthyroid mouse cerebellum as these were identified based on the GC–MS metabolomic analysis. Specifically, the positively and negatively significant metabolites of Table 2 have been positioned, appropriately color-coded (i.e., red for the positively and green for the negatively), in the network of the central carbon and amino acid metabolism reactions that was reconstructed based on currently available knowledge (see Materials and Methods). This map can be used to extract biologically relevant conclusions, which are discussed in the next section.

**Discussion**

In this work, we studied the primary metabolism of the Balb/cJ mouse cerebellum under prolonged AOH induced by a 64-day treatment with 1% potassium perchlorate in the drinking water of the animals, using GC–MS metabolomics for the acquisition of the polar metabolic profiles. Metabolomic analysis enabled the identification of 19 metabolites whose free pool size profile was discriminatory between the two groups. Seventeen of these significant metabolites were identified with decreased concentration in the hypothyroid compared to the euthyroid cerebellum (Table 2). This implies an overall decrease in the cerebellar metabolic activity due to AOH, manifested in major metabolic pathways and functions. The significance of these results is twofold. First, it provides strong evidence that the mammalian cerebellum is metabolically responsive to AOH. Second, the simultaneous measurement of metabolites that span a large part of the metabolic network enabled the integrated view of (a) pathways that were previously studied in the context of the AOH effect on brain metabolism, along with (b) reactions that would not have been investigated in connection to this condition based on currently available hypotheses. Thus, it became possible to identify and correlate within the same study and the specific brain region many of the alterations in the brain metabolic physiology that had been previously implicated with AOH. Furthermore, new directions for further investigation concerning AOH effects on brain metabolism were indicated, based on observed metabolic changes in mammalian cerebellum that had not been previously studied in connection to this disease, as it is discussed below.

Among the major observations in Figure 4 is the significant decrease in the tricarboxylic acid (TCA) cycle activity indicated by the pool size reduction of major TCA cycle intermediates and/or their products. TCA cycle is the main pathway of oxidative energy metabolism. Specifically, aspartate and fumarate are among the 4 most discriminatory metabolites between the hypothyroid and the euthyroid cerebella (Table 2). According to the known metabolic pathway stoichiometry and activity in the brain, the aspartate pool is considered at equilibrium with the oxaloacetate (OAA) pool. The significant decrease in the aspartate/OAA pool suggests decrease in the anaplerotic activity and a potential failure of the malate–aspartate shuttle. Moreover, in connection with the significant decrease in the fumarate pool, a decrease in the respiratory activity and oxidative phosphorylation can be inferred. The latter is in agreement with earlier studies showing thyroid hormones to affect the respiratory activity of adult brain mitochondria by directly impacting the succinate dehydrogenase reaction and...
the electron-transfer activity of the respiratory system. Decreased respiratory activity is consistent with the occurrence of oxidative stress in cerebellum under prolonged AOH, as a recent study implied for amygdala and hippocampus. Further studies focused on oxidative stress are required. It has to be noted that the observed lower TCA cycle activity contradicts a long-lasting notion that AOH does not affect the brain oxygen consumption rate, previously considered as the metabolic rate, and thus does not influence brain metabolism. In addition to the fact that the multicom-pound metabolic profile is a more accurate and sensitive sensor of changes in the brain metabolic physiology compared to a single measurement as the oxygen consumption rate, the difference in the AOH duration between the studies could not be excluded as a cause of this apparent contradiction. Indeed, in our study, the observed changes in the brain metabolic activity correspond to a prolonged treatment of the animals with KClO4.

The indication for lower TCA cycle activity in cerebellum due to AOH is further complemented by observations of lower activity in the glutamate/glutamine metabolism; GABA, urea, and 2-hydroxyglutarate are among the negatively significant metabolites (Figure 4). In the case when the animals 5E and 1H were excluded from the analysis (Supplementary File 4), the smaller biological variance of the investigated subgroups allowed for glutamate to be among the negatively significant metabolites too. Free urea, a brain organic osmolyte, has not been previously studied in relation to AOH effects on brain metabolism. Urea cannot be taken up from blood circulation; its formation in the brain is not related to nitrogen detoxification and the size of its free pool in the brain should depend only on its production rate from arginine/ornithine in the context of polyamine synthesis. Thus, its observed significant decrease suggests a decrease in the rate of polyamine synthesis in cerebellum due to AOH.

The significant decrease in the replenishment of the TCA cycle due to AOH is related to the observed significant decrease in the free glucose levels. Brain, having limited capacity to use alternative energy sources such as ketone bodies, depends strongly on glucose, originating mainly from plasma circulation,

Figure 3. (A) HCL (Euclidean distance) and (B) PCA of the profiles of the metabolites identified as significant from SAM for the significance threshold value d1 = 1.91 (see Figure 2 and Table 2). Both analyses validate that the profiles of these metabolites are discriminatory between the euthyroid (E-blue) and hypothyroid (H-purple) groups. PC1, PC2, and PC3 refer to the % profile variation from the original experimental space carried by principal components 1, 2, and 3, respectively. The abbreviation "inj-xx" refers to the n-th injection of a particular sample, corresponding thus to the n-th experimental replicate of this sample.
to meet its metabolic demands. The observed decrease in the free glucose levels agrees with previous reports of decreased peripheral glucose utilization due to AOH or decreased glucose uptake from the brain in neonatal hypothyroidism. However, in the present setup, it was not possible to determine the exact origin of the observed decrease. In general, hypothyroidism has been correlated with decline in the glucose metabolism; early in vitro studies indicated a decrease in the activity of the glycolytic enzymes hexokinase, phosphofructokinase, and pyruvate kinase in the hypothyroid brain.

Furthermore, based on the results of the present study, increase in the ketone body breakdown or amino acid oxidation or fatty acid β-oxidation, as alternative carbon and energy sources, can be precluded. All three are expected to replenish the acetyl-CoA and/or succinyl-CoA pools. In addition, ketone body breakdown requires succinyl-CoA to advance as shown in Figure 4. However, the significantly lower concentrations of aspartate/OAA and fumarate pools in the hypothyroid compared to the euthyroid cerebellum are not compatible with these scenarios.

Scyllo-inositol being among the most discriminatory metabolites between the euthyroid and the hypothyroid cerebella (2nd in the order of significance in Table 2) is an important observation. It is the first time that the particular metabolite has been directly implicated with the effect of AOH on brain metabolism in general and cerebellum physiology in particular. Actually, the measured peak area could in theory correspond to both scyllo- and chiro-inositol, because these two stereoisomers of inositol cannot be distinguished by GC-MS. In mammalian brain, however, this measurement can be attributed almost solely to scyllo-inositol; this is the only inositol stereoisomer that has been observed in considerable amount in this tissue, including the human brain. Rat, rabbit, and bovine brain have the ability to convert myo- to scyllo-inositol through the action of a specific epimerase.

Figure 4. The major changes in the metabolic physiology of the hypothyroid compared to the euthyroid adult mouse cerebellum, as these were identified based on the GC-MS metabolomic analysis. The positively and negatively significant metabolites of Table 2 are indicated in red- and green-filled boxes, respectively. The names of the metabolites, the peaks of which have been identified in at least one of the acquired metabolic profiles (see Supplementary File 2) are depicted in bold; if the metabolite RPA was included in the analysis after normalization and filtering (i.e., depicted in green background in Supplementary File 2), its name is also underlined. All orange are depicted names of the “essential” metabolites, which are only taken up from the blood circulation through the blood brain barrier, while in blue the names of the metabolites which can be taken up from the blood circulation and be also the products of reactions and interconversions within the brain. The neurotransmitters are depicted in purple rectangles. Dashed lines imply a series of reactions from the depicted to the corresponding product. DA, NA, and A stand for dopamine, noradrenaline, and adrenaline, respectively. *Note 1: See ref 41. *Note 2: Methionine is a precursor of S-adenosylmethionine (SAM), “a key methyl donor with a prominent role in the synthesis of several neurotransmitters and of creatine”.

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addition, scyllo-inositol may enter the brain\textsuperscript{54} using a common for myo- and scyllo-inositol transporter,\textsuperscript{58,59} but no measurements of the ratio between the \textit{in situ} production and the external transport rates have been reported. Although the presence of scyllo-inositol in mammalian brain has been known for a long time, its physiological role remains ambiguous. In humans, abnormally low brain scyllo-inositol levels have been observed in hepatic encephalopathy.\textsuperscript{66} In addition, in a mouse model of Alzheimer Disease, the animals that were treated with scyllo-inositol and not those treated with myo-inositol exhibited remarkable improvement in cognitive function, synaptic function, and lifespan.\textsuperscript{61} These studies, complemented with the present result in the context of AOH, indicate that the physiological role of scyllo-inositol on the cognitive functions of mammalian brain demands further in depth investigation, especially in cerebellum, the role of which in these functions has only recently been recognized.\textsuperscript{31}

Myo-inositol was also identified as negatively significant in hypothyroid compared to the euthyroid cerebella, but among the lower order of significance metabolites compared to scyllo-inositol (see Table 2). This might be attributed to the fact that myo-inositol is normally present in much higher concentrations in the brain than scyllo-inositol; thus, similar molar changes correspond to a stronger effect in the free scyllo-inositol than the free myo-inositol pool size. Myo-inositol is an important molecule in the physiology of the brain acting as osmolyte in the brain tissue\textsuperscript{44} and being a precursor for the synthesis of phosphatidylinositol, a major phospholipid in neural membranes and second messenger signaling systems.\textsuperscript{38} Therefore, the observed decrease in the cerebellar myo-inositol levels is in general agreement with reports that the \textit{in vivo} TH treatment of euthyroid rodents resulted in altered inositide composition of brain mitochondrial membranes.\textsuperscript{17} Moreover, alterations in the brain and cerebrospinal fluid (CSF) myo-inositol levels have been correlated with several psychopathological conditions,\textsuperscript{53} while administration of myo-inositol has been indicated beneficial for treating these disorders.\textsuperscript{62}

The observed significant decrease in the leucine concentration could be attributed to its decreased uptake rate from blood circulation. This decrease is consistent with the previously discussed decline in the activity of glutamate/glutamine metabolism; leucine acts as an amine-group donor for the synthesis of glutamate.\textsuperscript{53,64} Moreover, leucine is an essential branched chain amino acid, whose blood brain barrier (BBB) uptake rate has been considered a molecular index for the protein synthesis rate in the brain.\textsuperscript{66} The decreased leucine in conjunction with the decreased aspartate levels are consistent with decreased rate of protein synthesis in the hypothyroid compared to the euthyroid cerebellum. Validating this information with more specific studies on the rate of protein synthesis along with relevant proteomic analyses would be of interest.

Uracil is a major component of RNA synthesis, and the observed significant decrease in its concentration is in agreement with studies that have shown decreased levels of total RNA and rRNA in the brain of thyroidectomized adult rats.\textsuperscript{8} \(\beta\)-Alanine is the only naturally occurring \(\beta\)-amino acid, which can be synthesized from uracil in the liver and taken up from the brain through the BBB. \(\beta\)-alanine is a glycine agonist\textsuperscript{66} and a precursor of carnosine and its metabolites anserine and N-acetyl-carnosine.\textsuperscript{57} These histidine-based compounds along with homocarnosine, which requires GABA instead of \(\beta\)-alanine for its synthesis, have been known as endogenous antioxidants in the brain.\textsuperscript{67–69} The observed significant decrease in both the cerebellar \(\beta\)-alanine and GABA pools suggests a decrease in the synthesis of these antioxidants due to AOH. In addition, congenital defected \(\beta\)-alanine synthesis has been correlated with severe mental retardation, microcephaly, dystonic movements, and hypotonia,\textsuperscript{70} clinical symptoms that accompany congenital hypothyroidism and, some of them, AOH, too.

Threonate was one of the only two metabolites that were identified as positively significant under the particular pathological conditions; unfortunately, the most discriminatory positively significant metabolite peak area remains of unknown identity. The interest about threonate lies in the fact that it is one of the two products of ascorbate oxidation, but the other product, oxalate, was identified as negatively significant. Despite the significance of ascorbate as one of the most important antioxidant scavengers, the mechanisms and pathways in which it and its metabolites act on brain physiology have not been fully elucidated yet. In the brain, it enters in its oxidized form of dehydro-ascorbate (DHA), which crosses the BBB through glucose transporter 1;\textsuperscript{71} DHA may be reduced to ascorbate in the brain. No free ascorbate was detected in the cerebella of the euthyroid or hypothyroid groups. On the other hand, substantial concentrations of oxalate and threonate were measured in both groups. Of note, the mean concentration of the free oxalate in the euthyroid cerebellum was 4 times higher than that of threonate. The role of threonate in the brain has been mainly studied in conjunction with the antioxidant activity of ascorbate and it is not known if threonate in the brain could be converted to glycerate as it is the case in other eukaryotic systems.\textsuperscript{72} Oxalate has for long been considered a potentially toxic metabolic endpoint in mammalian metabolism and its synthesis route and exact functional role in mammalian cells have not been investigated in depth.\textsuperscript{73} Studies in other than brain cells have shown that oxalate could also be produced from other sources, mainly glyoxylate.\textsuperscript{73,74} However, these routes have not been explored in the mammalian brain. The observed substantial concentration of free oxalate in the euthyroid cerebellum suggests that the oxalate synthesis and use in the mammalian brain requires further investigation. Its concentration being much higher than that of threonate provides a strong indication that oxalate in the cerebellum is also produced from other than ascorbate sources.

This scenario is also consistent with the observed opposite effect of AOH on the oxalate and threonate pools. The increase in the threonate pool could be potentially attributed to an increased rate of DHA uptake under the particular hypoglycemic conditions, since DHA and glucose compete for the same transporter into the brain; in the opposite context, Chen et al.\textsuperscript{73} observed inhibition of DHA uptake in hyperglycemic tubular epithelial cells. If oxalate had been produced only from DHA, it would have also shown increase in its concentration in the hypothryoid compared to the euthyroid cerebellum. The observed decrease in its concentration, however, could be explained by a significant decrease in the rate of its production from its alternative precursors, including glyoxyxlate and oxaloacetate, due to AOH. Further specific investigations of oxalate synthesis, metabolism, and role in the brain are obviously required.

Conclusions

We carried out a metabonomic study of Balb/cJ mouse cerebellum under prolonged AOH induced by a 64-day
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Supplementary Files 3

References

Acknowledgment.

Supporting Information Available:

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