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Sex-comparative study of mouse cerebellum physiology under adult-onset hypothyroidism: The significance of GC–MS metabolomic data normalization in meta-analysis

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ABSTRACT

A systematic data quality validation and normalization strategy is an important component of the omic profile meta-analysis, ensuring comparability of the profiles and exclusion of experimental biases from the derived biological conclusions. In this study, we present the normalization methodology applied on the sets of cerebellum gas chromatography-mass spectrometry metabolic profiles of 124 days old male and female animals in an adult-onset-hypothyroidism (AOH) mouse model before combining them into a sex-comparative analysis. The employed AOH model concerns the monitoring of the brain physiology of Balb/cJ mice after eight-week administration of 1% w/v $KClO_4$ in the drinking water, initiated on the 60th day of their life. While originating from the same animal study, the tissues of the two sexes were processed and their profiles acquired and analyzed at different time periods. Hence, the previously published profile set of male mice was first re-annotated based on the presently available resources. Then, after being validated as acquired under the same analytical conditions, both profiles sets were corrected for derivatization biases and filtered for low-confidence measurements based on the same criteria. The final normalized 73-metabolite profiles contribute to the currently few available omic datasets of the AOH effect on brain molecular physiology, especially with respect to sex differentiation. Multivariate statistical analysis indicated one (unknown) and three (succinate, benzoate, myristate) metabolites with significantly higher and lower, respectively, cerebellum concentration in the hypothyroid compared to the euthyroid female mice. The respective numbers for the males were two and 24. Comparison of the euthyroid cerebellum metabolic profiles between the two sexes indicated 36 metabolites, including glucose, *myo*- and *scyllo*-inositol, with significantly lower concentration in the females versus the males. This implies that the female mouse cerebellum has been conditioned to smaller changes in its metabolic activity with respect to the pathways involving these metabolites compared to the male animals. In conclusion, our study indicated a much subtler AOH effect on the cerebellum metabolic activity of the female compared to the male mice. The leaner metabolic profile of the female mouse cerebellum was suggested as a potential factor contributing to this phenomenon.

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1. Introduction

Tissue metabolomic analysis is a multi-step procedure comprising both experimental and computational parts [1–3]. In meta-analysis of metabolomic data from various experiments, originating from the same or different labs, appropriate normalization and filtering of the profiles with respect to the potential differences in the experimental conditions at which they have been acquired is imperative [4,5]. These differences can be derived from any of

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the pre-analytical or analytical steps of the metabolomic analysis. The final normalized profiles can then be consistently used to draw biologically relevant conclusions. Otherwise, there is great risk of assigning biological significance to differences due to the experimental procedure. The need for standardized metabolomic data normalization is even higher in the case of animal studies, when one considers that each experiment corresponds to a different study group, probably from various housing facilities and animal colonies. Even in the cases at which the latter conditions are the same, considerable biological variation may be expected between animals of different births, grown at separate periods of the housing facility. To these experimental differences, one should add those associated with the pre-analytical steps of the metabolomic analysis and originate from (i) the animal sacrifice protocol, including the anesthesia procedures/agents used [6], (ii) the tissue perfusion protocol [7], (iii) the protocol and duration of tissue collection and handling before quenching, and (iv) the duration of frozen tissue storage before the metabolite extraction [8,9]. Even in the case that all measures have been taken to ensure that the experimental and pre-analytical conditions are the same among all samples, there may be variation among the acquired metabolic profiles originating from differences in the analytical steps of the metabolomic analysis procedure, i.e. the extraction protocol, the time of extraction, the metabolite derivatization protocol (if applicable), the profile acquisition method and the profile acquisition conditions [4,5,10]. Special quality control methods should be applied to ensure comparability between samples that differ in at least one of these parameters [11,12]. Finally, as the metabolomic data identification and quantification methods get improved and standardized, and the relevant databases become more extensive, combined analysis of metabolomic profiles from different acquisition periods may require re-annotation of the older profiles based on the currently available resources.

In this paper, we present a systematic normalization process in the context of a sex-comparative mouse model study of the prolonged adult onset hypothyroidism (AOH) effect on the cerebellum metabolic physiology based on gas chromatography-mass spectrometry (GC–MS) metabolomics. The mouse model of prolonged AOH concerns the chemical induction of the disease in Balb/cJ mice through the supply of 1% w/v $KClO_4$ in their drinking water for eight weeks, starting on the 60th day of their life (P60). The AOH chemical induction experiment was carried out simultaneously for the sibling male and female animals, including the animal sacrifice, tissue collection and storage on the 64th day of the treatment (124th postnatal day – P124), following the same protocols for both sexes in all steps. However, the metabolite extraction and metabolic profile acquisition of the tissues collected from the female mice were performed seven months after those for the male animals and the analysis of the profiles was recently completed. The entire metabolomic analysis for the male animals was previously published, providing at the time the first metabolic profiling study of brain physiology under AOH [13]. Thus, in order to collectively analyze the two sets of the cerebellum profiles, we had to (i) evaluate whether the two sets had been acquired under comparable measurement conditions using a relevant criterion for GC–MS metabolomic data presented in [4,14], (ii) re-annotate and re-analyze the profiles of the male mice according to the current peak libraries and (iii) correct for derivatization biases and filter out low confidence measurements following the same criteria [4,14] for both datasets. Finally, we comparatively investigated the two normalized sets using multivariate statistical analysis and discussed the results in the context of the reconstructed brain metabolic network to extract biologically relevant conclusions. The combined normalized dataset and the obtained results contribute to the currently limited omic studies of the AOH effect on brain physiology, especially with respect to the sex-comparative analyses.

2. Methods

2.1. Animal study design

The mouse model of prolonged AOH used in this study concerns the chemical induction of the disease in 60 days (P60) old Balb/cJ mice through the supply of 1% w/v $KClO_4$ in their drinking water for eight weeks [13]. The animal sacrifice and tissue isolation was carried out on the 64th day of treatment (P124). The cerebellum was rapidly weighed and frozen in liquid nitrogen after isolation to be finally stored at −80 °C until further analysis. The model is described in detail in the cerebellum metabolomic study of the male animals [13]. In this work, we analyzed the cerebellum metabolic profile of seven euthyroid (E) and seven hypothyroid (H) P124 Balb/cJ female mice originated from the same litter births as the male mice of the previous study. The experiment for the females was carried out in parallel and under the same housing conditions as for the males. The mice were weighed regularly (i.e. every three days) throughout the treatment period.

2.2. Metabolomic data acquisition, normalization & filtering

The GC–MS metabolic profile acquisition of the cerebellum tissue samples of the 14 P124 female mice was carried out following the same extraction, derivatization and data quantification protocols described in [13], but seven months after the processing of the male mouse tissues. The metabolic profile of each tissue sample was quantified at least thrice at derivatization times longer than 6 h, as justified elsewhere [4,14]; the experimental replicate X of a tissue sample will be referred to using the extension InjX (i.e., injection X). The peak identification was carried out based on the commercial NIST library (version 2.0), the Human Metabolome Database (version 3.6) and an in-house peak library. Each metabolite derivative was quantified based on its marker ion peak area. The raw metabolomic dataset comprised 139 marker ion peak areas (Supplementary File 1A). It is noted that our in house peak library has been enriched and the metabolite derivative peak annotation has in general been enhanced through expansion of the available databases, since the publication of the study for the male mice [13]. Thus, we re-annotated the cerebellum metabolomic dataset of the male animals based on the new information. Any modifications compared to the published marker ion set are described in Supplementary File 2 and the re-annotated raw metabolomic dataset of the male mouse cerebella is provided in Supplementary File 1A.

Metabolomic data validation, normalization and filtering were carried out as described in [4,14,15]. First, it was verified that all profiles of both the male and the female animals had been acquired under the same derivatization and GC–MS operational conditions, based on the constant ratio of the two derivative peak areas of the internal standard [U-^{13}C] – glucose, according to the relevant criterion presented in [4,14]. Having being validated as directly comparable, the two sets of profiles were first normalized with respect to the internal standard ribitol. Then, both sets of the relative (with respect to ribitol) peak area (RPA) profiles were corrected for derivatization biases affecting the amine-group containing metabolites (see [4,14]). Specifically, the algorithm described in [4,14] was employed to estimate an effective RPA for each metabolite of this type in every profile as the weighted sum of the RPAs of its multiple derivatives, using the same weights in both profile sets. Finally, low confidence measurements were filtered out of the normalized profiles using the same criteria for both profiles sets. Specific details for the normalization and filtering of the metabolomic dataset of the female animals and the re-annotated dataset of the male mice are provided in Supplementary File 3. The integrated normalized dataset after filtering comprises the marker ion RPA profiles of 73 metabolites, 32 of which are of

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