



# **Metabolomic Sexual Dimorphism of the Mouse Brain is Predominantly Abolished by Gonadectomy with a Higher Impact on Females**

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# Sex hormones fully control the metabolomic sexual dimorphism of the brain

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25 **Keywords:** brain, sexual dimorphism, metabolomics, lipidomics, sex hormones

26

27 **Abbreviations:** **Acc:** accuracy, **mVIP:** mean Variable Importance in Projection, **OPLS-DA:**

28 Orthogonal Projection to Latent Structures-Discriminant Analysis, **PC:** Phosphatidylcholine,

29 **PCA:** Principal Components Analysis, **Q<sup>2</sup><sub>C</sub>:** mean Cumulated Q-squared value, **sd:** standard

30 deviation, **SM:** sphingomyelin.

31   **Abstract**

32   We have recently highlighted the importance of sexual dimorphism of the mouse brain  
33   metabolome in addition to a high regional specificity between the frontal cortex, the  
34   cerebellum and the brain stem. To address the origin of this dimorphism, we performed  
35   gonadectomy on both sexes and reproduced the metabolomic study targeting 188 metabolites  
36   in the three brain regions. While sham controls, which underwent the same surgical procedure  
37   without gonadectomy, reproduced the regional sexual dimorphism of the metabolome  
38   previously identified, no sex difference was identifiable after gonadectomy, through both  
39   univariate and multivariate analyses. These experiments also made it possible to identify  
40   which sex was responsible for the dimorphism for 35 metabolites. The female sex contributed  
41   to the difference for more than 80% of them. Our results show that the sexual dimorphism of  
42   the cerebral metabolome is entirely controlled by the impregnation of sex hormones with a  
43   greater impact in females.

## 1. Introduction

The rapid emergence of personalized medicine in the past decades has urged the better consideration of sexual dimorphism in pre-clinical studies<sup>1,2</sup>. It remains important to thoroughly characterize the bases of this sexual dimorphism<sup>3</sup>. Many studies of the brain have found evidence of sex-specific differences in both humans and animals in various investigative fields including imaging<sup>4-8</sup>, epigenetics<sup>9</sup> and gene expression<sup>10,11</sup>.

Metabolomics is a robust approach for offering a large-scale view of both structural and functional metabolic alterations and has provided evidence of a strong sexual dimorphism in both humans and animals<sup>3</sup>.

We recently reported a high level of sexual dimorphism in the mouse brain metabolome, as well as a high regional specificity of the frontal cortex, brain stem and cerebellum<sup>12</sup>. This study was performed by carrying out a targeted metabolomics analysis of 3-month-old wild type C57BL-6J male (n = 20) vs. female (n = 20) mice. More than half of the 129 accurately measured metabolites disclosed a sexual dimorphism, i.e. a sex-specific variation of concentration in the whole brain, while only 8% of those (hydroxyproline, creatinine, hexoses, tryptophan, threonine and lysoPC.a.C18.2) were commonly involved in the three cerebral regions. In contrast, 71% of the dimorphic metabolites, including phosphatidylcholines, lysophosphatidylcholines, sphingomyelins, acylcarnitines, amino acids, biogenic amines and polyamines, presented a sexual dimorphism specific to only one brain region, uncovering the high regional specificity of cerebral sexual dimorphism in mice. The discriminating metabolites are considered key players in energetic metabolism (hexoses, acylcarnitines and creatinine), neurotransmission and neuromodulation (phosphatidylcholines, proline, glycine, serine, threonine, lysine, tryptophan, tyrosine, histamine, histidine, carnosine and polyamines), membrane structure and cell composition (phosphatidylcholines,

69 lysophosphatidylcholines and sphingomyelins), myelin metabolism (lysophosphatidylcholines  
70 and sphingomyelins), oxidative stress (methionine, sulfoxide methionine, carnosine and  
71 polyamines), folate metabolism (serine and methionine) and finally cell signaling system  
72 (polyamines).

73

74 A consideration of the sexual differences in the clinical expression existing for most  
75 neurological diseases <sup>13</sup> and the precise and exhaustive mapping of the sexual dimorphism of  
76 the cerebral metabolome, including the evaluation of the respective contribution of hormonal,  
77 chromosomal, epigenetic and developmental processes in defining gender differences, will  
78 contribute to better understanding these differences <sup>14</sup>. In this article, we address the origin of  
79 the brain's metabolic dimorphism based on hormonal impregnation after performing complete  
80 gonadectomy of female and male mice and the assessment of 188 metabolites by mass  
81 spectrometry <sup>15</sup>.

## 82 **2. Results**

83 Metabolite concentrations in the frontal cortex (weight-normalized) and the brain stem and  
84 cerebellum (total protein-normalized) are available in **Supplementary Table S1** (15,096  
85 measurements). As expected, due to the extraction protocol, the unsupervised analysis of  
86 datasets showed a slight batch effect in cerebellum samples, which was withdrawn after  
87 ComBat adjustment (**Supplementary Figure 1**).

88

89 OPLS-DA models allowed a robust discrimination of sham female *vs.* male samples for the  
90 three brain tissues (**Table 1**) with mean Q2<sub>C</sub> values higher than the commonly accepted  
91 threshold found in the literature (Q2<sub>C</sub> > 0.4 <sup>16</sup>) and mean accuracy on test sets higher than

92 90%. By contrast, models computed for the comparisons between castrate groups showed  
93 poor prediction ability, with  $Q^2_C$  lower than 0.4 and higher variance of accuracy on test sets.

94  
95 Univariate analyses provided evidence of the disappearance of sexual dimorphism after  
96 gonadectomy (**Figure 1**). Without Benjamini-Hochberg adjustment, 21 metabolites showed a  
97 significant sex dimorphism ( $p < 0.01$ ) in mice without gonadectomy: histamine and five  
98 glycerophospholipids in the frontal cortex; creatinine, leucine, valine, two acylcarnitines, five  
99 glycerophospholipids and two sphingomyelins in the cerebellum; and carnosine, creatinine  
100 and hexoses in cerebellum and transhydroxyproline in all regions. After Benjamini-Hochberg  
101 adjustment, only transhydroxyproline in the frontal cortex and brain stem showed a significant  
102 sex dimorphism in those mice ( $q < 0.01$ ). In contrast, none of those metabolites showed a  
103 significant sex dimorphism in the gonadectomized mice ( $p < 0.01$ ).

104  
105 Besides the fact that gonadectomies demonstrated the hormonal origin of the metabolic sexual  
106 dimorphism in the brain, they also showed that most variations of the dimorphic metabolites  
107 occur in females. Indeed, **Figure 1** clearly shows that concentrations of 31 metabolites vary in  
108 females with ovariectomy, up or down compared to male levels, which themselves do not  
109 vary with orchietomy: histamine (frontal cortex), carnosine (brain stem and cerebellum),  
110 creatinine (brain stem and cerebellum), hexoses (brain stem and cerebellum), leucine (brain  
111 stem), valine (brain stem), 20 phosphatidylcholines (19 in brain stem, one in frontal cortex),  
112 one lysophosphatidylcholine (frontal cortex), two sphingomyelins (brain stem) and two  
113 acylcarnitines (brain stem). In contrast, only five metabolites (trans-hydroxyproline or t4-OH-  
114 Pro (all tissues), tryptophan (frontal cortex), two phosphatidylcholines (one in frontal cortex  
115 and one in cerebellum) and one lysophosphatidylcholine (brain stem)) tended to vary in males  
116 after castration, without variations in females.

117  
118 Interestingly, phosphatidylcholine PC aa 34:3 shows a significant decrease in the female brain  
119 stem after ovariectomy ( $p < 0.01$ ,  $q > 0.01$ ), but increased in the male frontal cortex after  
120 orchidectomy.

121  
122 Surprisingly, a sexual dimorphism appeared after gonadectomy for three metabolites:  
123 asparagine, threonine and PC aa C34:1 ( $p < 0.01$ ,  $q \geq 0.01$ ). Asparagine, which did not show  
124 sex-specific differences in sham groups, increased in castrated female frontal cortex.

125 Similarly, a higher concentration of PC aa C34:1 in females appeared after gonadectomies in  
126 the cerebellum. In contrast, the castration led to a higher concentration of threonine in the  
127 frontal cortex and the brain stem in castrated males, compared to castrated females.

128  
129 To compare the sex-discriminating metabolites between the sham groups with those from our  
130 previous study, we compared the metabolites presenting a VIP  $> 0.7$  in the present study with  
131 those presenting a VIP  $> 1$  in the previous study (**Supplementary Figure 2**). The lower VIP  
132 threshold was chosen for two reasons: firstly, in order to take into account the lower statistical  
133 power of the present study (10/10 animals per group versus 20/20 previously)<sup>12</sup>; and  
134 secondly, because the main focus of this study consisted in comparing the metabolomic  
135 profiles, i.e. whether the metabolites concentrations vary in the same direction, and their  
136 relative importance for sex prediction. Furthermore, in this study, only metabolites with  
137 significantly different values between females and males ( $p < 0.05$ ) were used to train the  
138 OPLS-DA models.

139  
140 As expected, the most discriminating metabolites identified in the first study were found again  
141 in this second one, as for instance, the increase in male of trans-hydroxyproline and hexoses



in all brain regions under consideration, whereas not all the metabolites with lower VIP were found in this second study as shown in the Venn diagrams of **Supplementary Figure 2**. Furthermore, all metabolites with a VIP/mVIP higher than the threshold in at least one study, except one (PC aa C32:0 in the frontal cortex), showed concentration varying in the same direction according to the sex in both studies. Finally, this study confirms the presence of a large cluster of glycerophospholipids specifically increased in the female brain stem, with 29, 42 and 23 dimorphic glycerophospholipids in the previous study, in the current study and common throughout both studies, respectively.

### **3. Discussion**

Gonadectomy experiments demonstrated the almost exclusive hormonal origin of the metabolic sexual dimorphism of the brain, since unlike in sham controls, multivariate sex predictions became impossible after gonadectomy, while univariate analyses discriminated only three metabolites remaining after castrations in both sexes. Why asparagine, threonine and phosphatidylcholine PC aa C34:1 appeared dimorphic after gonadectomies remains unanswered. Non-hormonal causes, such as the influence of sexual chromosomes, epigenetics or brain development could be involved and counterbalanced by sex hormones in sham mice, maintaining similar levels of these metabolites in non-gonadectomized mice. Interestingly, the specific significant decrease of phosphatidylcholine PC aa 34:3 concentration in the female brain stem, compared to its increased concentration in the male frontal cortex, shows that the hormonal response is not only brain region-specific but it may even act in the opposite direction according to the brain region.

Among the 40 discriminant metabolites observed in this study, 35 allowed to unambiguously identify which of the two sexes contribute to the dimorphism. However, assigning those variations to an abundance of either androgens or estrogens is tricky, due to the wide effects

of androgens in female and estrogens in male mammals<sup>17</sup>. Indeed, mouse ovaries and testes both produce testosterone, while multiple tissues, including the brain, express the aromatase enzyme, allowing a local conversion to  $\beta$ 17-estradiol<sup>18–20</sup>. However, the fact that the sexual dimorphism of more than 80% of the discriminant metabolites is predominantly due to females supports the concept that brain regions are more sensitive to sex hormones in females than males. In conclusion, our study demonstrates the quasi-exclusive influence of sex hormones, particularly in females, on the sexual dimorphism of the brain metabolome.

#### **4. Materials and Methods**

The study design is shown in **Figure 2**.

##### *4.1. Animal procedures*

All experiments were performed in accordance with the European Community Guiding Principles for the care and use of animals (Directive 2010/63/UE; Décret n°2013-118) after approval by the French Ministry of Higher Education and Research and the attached Ethics Committee (authorization number 13908). Animal procedures, including manipulations, housing, environment and feeding were carried out according to the procedures we have described in an earlier article<sup>12</sup>. Forty C57BL/6 mice were divided into four groups of ten animals: sham female, castrate female, castrate male and sham male. Mice underwent surgery at 3 months old: ovariectomy for mice in the female castrate group, orchiectomy for mice in the male castrate group and sham surgery without gonads removal for both female and male sham groups. After a rest period of one month, allowing a full recovery following surgery and a complete clearance of sex hormones, animals were euthanized and dissected in order to collect frontal cortex, brain stem and cerebellum samples, as previously described<sup>12</sup>.

##### *4.2. Extraction and metabolomic analysis*

Samples were extracted as described previously<sup>12</sup>. Frontal cortex samples were extracted using 3 µl/mg of cold methanol after weighing. Due to the low weight of brain stem and cerebellum samples, hindering its accurate measuring, those samples were initially extracted using 100 µl of deionized water in order to determine the total protein concentration, for normalization between samples. Water extracts were then re-extracted with a constant volume of 200 µl methanol, evaporated and dissolved in 50 µl methanol before mass spectrometry analysis. Metabolomic analyses were carried out by targeted mass spectrometry, using the p180 kit from Biocrates (Biocrates Life Sciences Ag, Innsbruck, Austria) and a Q-Trp 550 mass spectrometer (AB Sciex LLC, Redwood City CA, USA). This standardized technology, using quality controls and quantification curves, allows for the quantification of 188 metabolites including hexoses, acylcarnitines, amino acids, biogenic amines, phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins.

#### 4.3. Statistical workflow

Datasets from every tissue were processed independently through the same workflow. Metabolites with more than 30% of values lying outside the detection limits were removed after a Chi-square test to ensure the absence of a link between sex and distribution of concentrations in and out of the detection limits. For brain stem and cerebellum samples, concentrations were normalized by the total protein concentration of each sample. Since our extraction protocol was realized in batches of twenty samples and included a water-extraction step for total protein concentration measurement (*i.e.* before metabolism quenching), datasets were adjusted for batch effects using ComBat<sup>21</sup>. Unsupervised Principal Components Analysis (PCA) was used to identify potential outliers and assess batch effect, before and after ComBat adjustment. Metabolite concentrations were then standardized by mean subtraction and standard deviation division for multivariate analyses.

#### 4.4. Univariate analyses

Non-parametric Wilcoxon signed-rank tests were computed between sham females and males, castrate females and males, sham and castrate females and sham and castrate males.  $p$ -values were adjusted into  $q$ -values using the Benjamini-Hochberg method to take into account the false discovery rate due to multiple analyses.

#### 4.5. Multivariate models

Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) models were used to predict sex according to metabolite concentrations, for both sham (females vs. males) and castrate (females vs. males) groups, in order to assess the sexual dimorphism for both shams and castrates. Models were trained only with metabolites with a  $p$ -value  $< 0.05$  between groups. To ensure the reproducibility of our results, we performed a 100-iteration bootstrapping without replacement for each model, with 66/33% training/test set stratified partitioning. The robustness of the OPLS-DA models was assessed with their mean cumulated Q-squared value (mean  $Q^2_c$ ) and their mean overall accuracy (mean Acc) on validation sets over all iterations. The importance of each feature regarding sex or tissue discrimination was then assessed by retrieving the mean Variable Importance in Projection (mVIP) of each compound for each model<sup>22</sup>. Metabolites were then compared using mVIP and fold-change for sex prediction.

#### 4.6. Software

All data processing, statistical analysis and graphical work were carried out using the R software (version 3.4.1, 64-bit)<sup>23</sup>. PCA values were computed using the FactoMineR R package (version 1.39)<sup>24</sup>. The OPLS-DA models were computed using the Bioconductor ropls R package (version 1.10.0)<sup>25</sup>. Images were conceived using GNU Image Manipulation Program (GIMP) software (version 2.10).