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

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24

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, **Q²_c:** 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.

44 **1. Introduction**

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

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

53

54 We recently reported a high level of sexual dimorphism in the mouse brain metabolome, as
55 well as a high regional specificity of the frontal cortex, brain stem and cerebellum ¹². This
56 study was performed by carrying out a targeted metabolomics analysis of 3-month-old wild
57 type C57BL-6J male (n = 20) vs. female (n = 20) mice. More than half of the 129 accurately
58 measured metabolites disclosed a sexual dimorphism, i.e. a sex-specific variation of
59 concentration in the whole brain, while only 8% of those (hydroxyproline, creatinine,
60 hexoses, tryptophan, threonine and lysoPC.a.C18.2) were commonly involved in the three
61 cerebral regions. In contrast, 71% of the dimorphic metabolites, including
62 phosphatidylcholines, lysophosphatidylcholines, sphingomyelins, acylcarnitines, amino acids,
63 biogenic amines and polyamines, presented a sexual dimorphism specific to only one brain
64 region, uncovering the high regional specificity of cerebral sexual dimorphism in mice. The
65 discriminating metabolites are considered key players in energetic metabolism (hexoses,
66 acylcarnitines and creatinine), neurotransmission and neuromodulation (phosphatidylcholines,
67 proline, glycine, serine, threonine, lysine, tryptophan, tyrosine, histamine, histidine, carnosine
68 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 ¹³ 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 ¹⁴. 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 ¹⁵.

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 $Q2_C$ values higher than the commonly accepted
91 threshold found in the literature ($Q2_C > 0.4$ ¹⁶) 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 $Q2_C$ 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 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)¹²; 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

142 in all brain regions under consideration, whereas not all the metabolites with lower VIP were
143 found in this second study as shown in the Venn diagrams of **Supplementary Figure 2**.
144 Furthermore, all metabolites with a VIP/mVIP higher than the threshold in at least one study,
145 except one (PC aa C32:0 in the frontal cortex), showed concentration varying in the same
146 direction according to the sex in both studies. Finally, this study confirms the presence of a
147 large cluster of glycerophospholipids specifically increased in the female brain stem, with 29,
148 42 and 23 dimorphic glycerophospholipids in the previous study, in the current study and
149 common throughout both studies, respectively.

150 **3. Discussion**

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

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

166 of androgens in female and estrogens in male mammals¹⁷. Indeed, mouse ovaries and testes
167 both produce testosterone, while multiple tissues, including the brain, express the aromatase
168 enzyme, allowing a local conversion to β 17-estradiol¹⁸⁻²⁰. However, the fact that the sexual
169 dimorphism of more than 80% of the discriminant metabolites is predominantly due to
170 females supports the concept that brain regions are more sensitive to sex hormones in females
171 than males. In conclusion, our study demonstrates the quasi-exclusive influence of sex
172 hormones, particularly in females, on the sexual dimorphism of the brain metabolome.

173 **4. Materials and Methods**

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

175 *4.1. Animal procedures*

176 All experiments were performed in accordance with the European Community Guiding
177 Principles for the care and use of animals (Directive 2010/63/UE; Décret n°2013-118) after
178 approval by the French Ministry of Higher Education and Research and the attached Ethics
179 Committee (authorization number 13908). Animal procedures, including manipulations,
180 housing, environment and feeding were carried out according to the procedures we have
181 described in an earlier article¹². Forty C57BL/6 mice were divided into four groups of ten
182 animals: sham female, castrate female, castrate male and sham male. Mice underwent surgery
183 at 3 months old: ovariectomy for mice in the female castrate group, orchiectomy for mice in
184 the male castrate group and sham surgery without gonads removal for both female and male
185 sham groups. After a rest period of one month, allowing a full recovery following surgery and
186 a complete clearance of sex hormones, animals were euthanized and dissected in order to
187 collect frontal cortex, brain stem and cerebellum samples, as previously described¹².

188 *4.2. Extraction and metabolomic analysis*

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

201 *4.3. Statistical workflow*

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

213 *4.4. Univariate analyses*

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

218 *4.5. Multivariate models*

219 Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) models were
220 used to predict sex according to metabolite concentrations, for both sham (females vs. males)
221 and castrate (females vs. males) groups, in order to assess the sexual dimorphism for both
222 shams and castrates. Models were trained only with metabolites with a *p*-value < 0.05
223 between groups. To ensure the reproducibility of our results, we performed a 100-iteration
224 bootstrapping without replacement for each model, with 66/33% training/test set stratified
225 partitioning. The robustness of the OPLS-DA models was assessed with their mean cumulated
226 Q-squared value (mean Q_{2c}) and their mean overall accuracy (mean Acc) on validation sets
227 over all iterations. The importance of each feature regarding sex or tissue discrimination was
228 then assessed by retrieving the mean Variable Importance in Projection (mVIP) of each
229 compound for each model²². Metabolites were then compared using mVIP and fold-change
230 for sex prediction.

231 *4.6. Software*

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