**Motor function deficits in the estrogen receptor beta knockout mouse: Role on excitatory neurotransmission and myelination in the motor cortex**

Mukesh Kumar Varshney1, Nancy Yiu-Lin Yu1, Shintaro Katayama1, Xin Li2, Tianyao Liu2, Wan-Fu Wu3, Virpi Töhönen1,4, Kaarel Krjutškov1,5,6, Juha Kere1,6, Xiaotang Fan2, José Inzunza1, Jan-Åke Gustafsson1,4, Ivan Nalvarte1\*

1Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden

2Department of Developmental Neuropsychology, School of Psychology, Third Military Medical University, Chongqing, China

3Center for Nuclear Receptors and Cell Signaling, University of Houston, Houston, TX, USA

4Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

5Competence Centre on Health Technologies, Tartu, Estonia

6Folkhälsan Research Institute, Helsinki, and Stem Cells and Metabolism Research Program, University of Helsinki, Helsinki, Finland

Short Title: Loss of ERβ is associated with motor function deficits

\*Corresponding Author

Ivan Nalvarte

Department of Biosciences and Nutrition

Karolinska Institutet

Hälsovägen 7C, Neo

Huddinge, SE-14157, Sweden

Tel: +46-8-52481148

E-mail: ivan.nalvarte@ki.se

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**Supplemental Fig. 1. STRT RNA-Sequencing analysis of left and right WT and BERKO primary motor cortex.** (**A**) Heatmap of differently expressed genes between WT and BERKO left and right primary motor cortices. Each column represents data from one mouse (n=5 BERKO, 6 WT). Levels are based on log2 mean signal intensities, red and blue denote high and low expression levels respectively. (**B**) Venn diagrams showing number of commonly upregulated or downregulated genes between left and right motor cortices. RT-qPCR analysis of genes involved in regulation of dendrite extension (**C**), synapse (**D**), and the myelin sheath (**E**). Differences in gene expression between left or right BERKO M1 cortex were analyzed relative to respective left or right WT M1 cortex gene expression (dashed line, n=3). The main effect of hemisphere was analyzed by using a 2-way ANOVA with Bonferroni’s correction for multiple comparisons (main effect of hemisphere in C: *F*(1,16) = 2.222, *p* = 0.1555; in D: *F*(1,32) = 39.98, *p* < 0.001; in E: *F*(1,16) = 7.324, *p* = 0.01566. Effect of gene in C: *F*(3,16) = 30.01, *p* < 0.001; in D: *F*(7,32) = 154.9, *p* < 0.001; in E: *F*(3,16) = 12.33 *p* < 0.001. Interaction between hemisphere and gene in C: *F*(3,16) = 1.605 *p* = 0.2274; in D: *F*(7,32) = 40.75, *p* < 0.001; in E: *F*(3,16) = 1.139, *p* = 0.3632. \*\*\* *p* < 0.001).

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**Supplemental Fig. 2. ERβ expression in M1 cortex**. (**A**) RT-qPCR analysis of ERβ expression relative to *Rplp0* housekeeping gene expression in WT and BERKO M1 cortex (n=13 replicates from 3 mice) (t-test WT vs BERKO *t* = 8.174, df = 4, *p* = 0.0012). Representative images of ERβ immunofluorescence staining in WT M1 cortex. Although few, both NeuN+ neuronal cells (**B**) and NeuN- non-neuronal cells (**C**) were found to express ERβ (arrowheads). Scale bars: 50µm. *P* values were obtained using Student’s *t*-test (A), \*\* *p* < 0.01 compared to WT. Data represent means ±SEM.

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**Supplemental Fig. 3. ERβ expression around M1 cortex**. (**A**) Representative image of ERβ staining at low magnification of WT cortex. Boxed areas denote magnified areas of upper (1) and deeper (2) layers of M1 cortex, as well as corpus callosum (3). Negative control of ERβ staining in BERKO cortex (**B**) and without primary antibody in WT cortex (**C**). Scale bars: 100 µm.

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**Supplemental Fig. 4. Subtle differences between left and right M1 cortex or in prefrontal cortex synapses in WT and BERKO brains.** Quantification of (**A**) number of excitatory synapses per µm2 (2-way ANOVA of main effect of genotype *F*(1, 8) = 0.9955, *p* = 0.3476; hemisphere *F*(1,8) = 0.0361, *p* =0.8540; and interaction between genotype and hemisphere *F*(1,20) = 1.646, *p* = 0.2355) and (**B**) vesicles per µm2 excitatory synapse (2-way ANOVA of main effect of genotype *F*(1,8) = 4.327, *p* = 0.0711; hemisphere *F*(1,8) = 0.2648, *p* = 0.6208; and interaction between genotype and hemisphere *F*(1,8) = 4.893, *p* = 0.0579) in prefrontal cortex of WT and BERKO mice. Quantification of (**C**) mean postsynaptic intensity (2-way ANOVA of main effect of genotype *F*(1,8) = 1.392, *p* = 0.2720; hemisphere *F*(1,8) = 0.5977, *p* =0.4617; and interaction between genotype and hemisphere *F*(1,8) 1.024, *p* = 0.7571) (AU, arbitrary units) and (**D**) number of inhibitory symmetric synapses per µm2 (2-way ANOVA of main effect of genotype *F*(1,8) = 35.53, *p* < 0.001; hemisphere *F*(1,8) = 0.0038, *p* =0.9526; and interaction between genotype and hemisphere *F*(1,8) = 0.1296, *p* = 0.7281) in prefrontal cortex of WT and BERKO mice. (**E**) Number of vesicles per µm2 inhibitory synapse (2-way ANOVA of main effect of genotype *F*(1,8) = 4,327, *p* = 0.0711; hemisphere *F*(1,8) = 0.2648, *p* = 0.6208; and interaction between genotype and hemisphere *F*(1,8) = 4.893, *p* = 0.0579 in prefrontal cortex of WT and BERKO mice. (**F**) number of excitatory synapses per µm2 (2-way ANOVA of main effect of genotype *F*(1,20) = 9.208, *p* = 0.0065; hemisphere *F*(1,20) = 2.295, *p* =0.1454; and interaction between genotype and hemisphere *F*(1,20) = 0.9776, *p* = 0.3346) and (**G**) vesicles per µm2 excitatory synapse (2-way ANOVA of main effect of genotype *F*(1,20) = 72.58, *p* < 0.001; hemisphere *F*(1,20) = 11.27, *p* = 0.0031; and interaction between genotype and hemisphere *F*(1,20) = 1.975, *p* = 0.1752) in M1 cortex of WT and BERKO mice. Quantification of (**H**) mean postsynaptic intensity (2-way ANOVA of main effect of genotype *F*(1,20) = 1.199, *p* = 0.2865; hemisphere *F*(1,20) = 4.340, *p* =0.0503; and interaction between genotype and hemisphere *F*(1,20) = 2.901, *p* = 0.1040) (AU, arbitrary units) and (**I**) number of inhibitory symmetric synapses per µm2 (2-way ANOVA of main effect of genotype *F*(1,16) = 40.03, *p* < 0.001; hemisphere *F*(1,16) = 1.108, *p* =0.3082; and interaction between genotype and hemisphere *F*(1,16) = 0.601, *p* = 0.4497) in M1 cortex of WT and BERKO mice. (**J**) Number of vesicles per µm2 inhibitory synapse (2-way ANOVA of main effect of genotype *F*(1, 20) = 0.4073, *p* = 0.5306; hemisphere *F*(1,20) = 0.1549, *p* =0.6980; and interaction between genotype and hemisphere *F*(1,20) = 0.02176, *p* = 0.8842) in M1 cortex of WT and BERKO mice. *P* values were obtained using 2-way ANOVA with Tukey’s correction for multiple comparisons. Data represent means ±SEM (n=3 in A-E, n=5 in I, and n=6 in F-H, J).

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**Supplemental Fig. 5. Magnified images of calretinin immunoreactive cells.** Representative high-contrast magnified images of Calretinin+ neurons in WT and BERKO right M1 cortex. Insert: Calretinin immunofluorescence at low contrast; yellow box: area of quantification, 1 mm2. Blue box: magnified area representing part of quantified cells. Scale bar: 100 µm.

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**Supplemental Fig. 6. Morphological analysis of the BERKO motor and prefrontal cortex.** (**A**) Quantification of Mbp staining in left (L) and right (R) WT and BERKO M1 cortex layer III by mean gray intensity (AU, arbitrary units) (2-way ANOVA of main effect of genotype *F*(1,12) = 24.64, *p* < 0.001; hemisphere *F*(1,12) = 0.4222, *p* = 0.5281; and interaction between genotype and hemisphere *F*(1,12) = 0.6306, *p* = 0.4426). (**B**) Representative images of Plp1 staining of WT and BERKO M1 cortex (at layer III, scale bar: 20 µm). Right panel, quantification of Plp1 staining in the left and right WT and BERKO M1 cortex (2-way ANOVA of main effect of genotype *F*(1,8) = 38.39, *p* < 0.001; hemisphere *F*(1,8) = 3.891, *p* = 0.0840; and interaction between genotype and hemisphere *F*(1,8) = 1.103, *p* = 0.3242). (**C**) Representative images of NeuN, and DAPI staining in WT and BERKO cortex show disorganized neuronal layering (scale bars: 200 µm). (**D**) Nissl staining in the prefrontal cortex show no difference in neuronal layering (scale bars: 200 µm). (**E**) Quantification of average myelin g-ratios in left and right WT and BERKO M1 cortex (2-way ANOVA of main effect of genotype *F*(1,16) = 20.33, *p* < 0.001; hemisphere *F*(1,16) = 0.0530, *p* = 0.8209; and interaction between genotype and hemisphere *F*(1,16) = 0.1423, *p* = 0.7109. (**F**) Representative images of axonal myelination in WT and BERKO prefrontal cortex (Scale bars: 1µm). Quantification of (**G**) average myelin g-ratios in WT and BERKO prefrontal cortex (t-test WT vs BERKO *t* = 1.513, df = 4, *p* = 0.2049) and (**H**) g-ratios as a function of axon diameter length. The scatter plot depicts r2-values and slopes (WT, blue, n=83 from 3 mice; BERKO, red, n=82 from 3 mice). Linear regression analysis show that there is no significant difference between the slopes (*F*(1,161) = 3.7431, *p* = 0.0548), and no difference between the slope elevations (*F*(1,162) = 2.3879, *p* =0.1242). *P* values were obtained using 2-way ANOVA with Tukey’s correction for multiple comparisons (A, B, E) or Student’s *t*-test (G). Data represent means ±SD (A, B) or ±SEM (E, G) (n=4 in A; n=5 in E; and n=3 in B, G, H; ).

**Supplemental Fig. 7. ERβ expression in OPCs and mature OLs.** (**A**) Quantification of Olig2+ cells per mm2 in 3 WT and 3 BERKO M1 left and right cortices. Data was analyzed by 2-way ANOVA of main effect of genotype *F*(1,8) = 10.90, *p* = 0.0108; hemisphere *F*(1,8) = 0.2225, *p* = 0.6497; and interaction between genotype and hemisphere *F*(1,21) = 0.02061, *p* = 0.8894). Representative images of expression of (**B**) ERβ and Olig2 in WT and BERKO OPC cultures (n=3). (**C**) ERβ and CNPase (marker of OLs) expression in WT and BERKO OL cultures (n=3). ERβ staining was found in both OPCs and OLs as well as in non-OPC and non-OL cells. Scale bars: 50 µm. *P* values were obtained using 2-way ANOVA with Tukey’s correction for multiple comparisons (A), \**p* < 0.05. Data represent means ±SEM.