Title: Efficacy of vafidemstat in experimental autoimmune encephalomyelitishighlights the KDM1A/RCOR1/HDAC epigenetic axis in multiple sclerosis

Authors: Fernando Cavalcanti[1], Elena Gonzalez-Rey[2], Mario Delgado[2], Leyre Mestre[3], Carmen Guaza[3], Michele MP Lufino[1], Jordi Xaus[1], Cristina Mascaró[1], Serena Lunardi[1], Natalia Sacilotto[1], Paola Dessanti[1], David Rotllant[1], Xavier Navarro[4], Mireia Herrando-Grabulosa[4], Carlos Buesa[1] and Tamara Maes[1]

[1] Oryzon Genomics, S.A. Carrer Sant Ferran 74, 08940 Cornellà de Llobregat, Spain.

[2] Institute of Parasitology and Biomedicine Lopez-Neyra, IPBLN-CSIC, Granada, Spain.

[3] Department of Functional and Systems Neurobiology, Cajal Institute (CSIC), Madrid, Spain.

[4] Departament de Biologia Cel.lular, Fisiologia i Immunologia, Institut de Neurociències, Universitat Autònoma de Barcelona, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain.

Additional File 3

Additional Figures

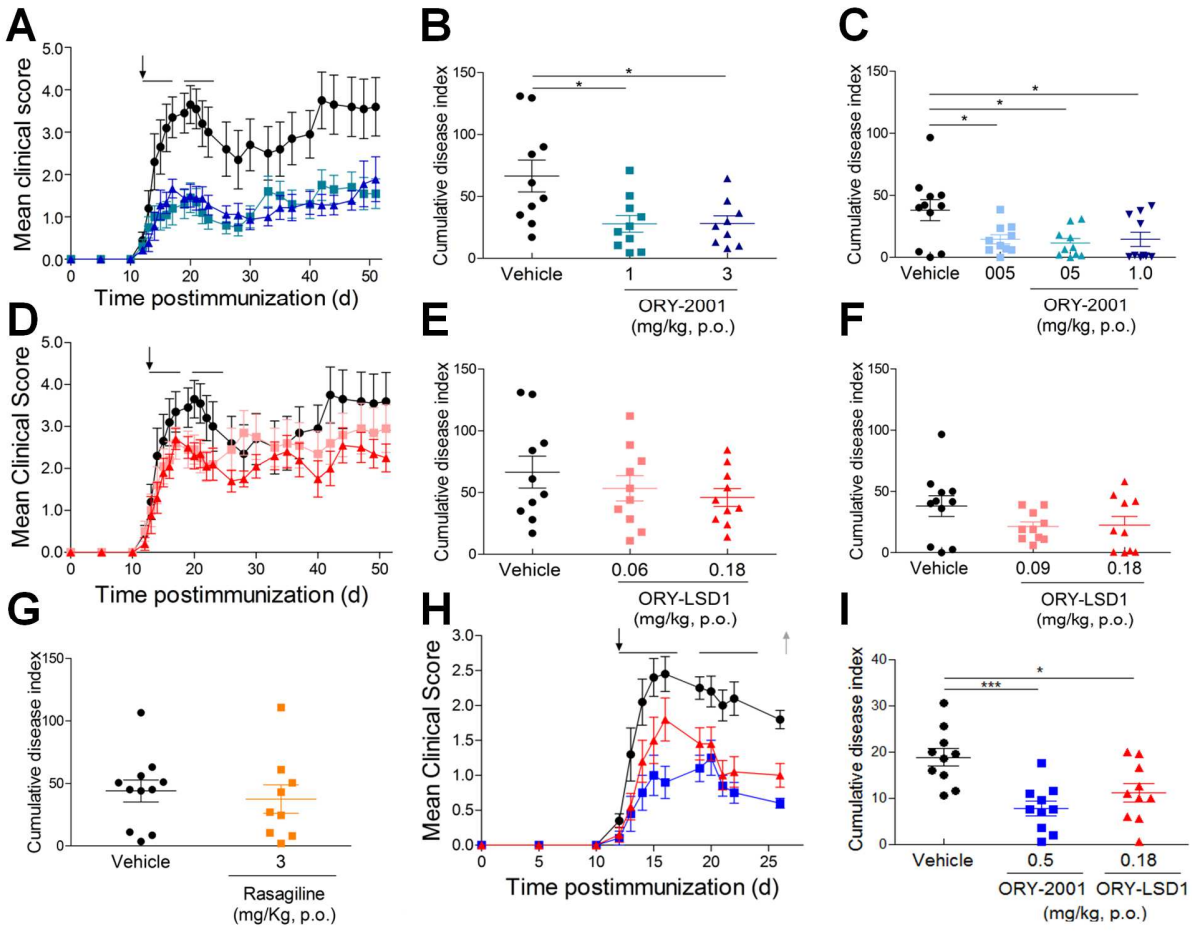

Figure A1. Therapeutic effects of ORY-2001 in EAE mice

(**A**)The effects of ORY-2001 on the clinical score of EAE mice during the chronic phase. The clinical score was monitored before, during, and after two weeks of treatment (five consecutive days per week) with ● (black) Vehicle or ORY-2001 (blue) at ■ 1.0 mg/kg and ▲3.0 mg/kg (N = 9-10 mice / group). Cumulative disease index of Vehicle and ORY-2001 treatment at (**B**) 1 and 3 mg/kg and (**C**) 0.05, 0.1 and 0.5 mg/kg. (**D**) The effects of ORY-LSD1 on the clinical score of EAE mice during the chronic phase. The clinical score was monitored before, during, and after two weeks of treatment (five consecutive days per week) with ● (black) Vehicle or ORY-LSD1 (red) at ■ 0.06 mg/kg and ▲0.18 mg/kg (N = 10 mice / group). Cumulative disease index of Vehicle and ORY-LSD1 treatment at (**E**) 0.06 and 0.18 mg/kg and (**F**) 0.09 and 0.18 mg/kg. (**G**) Cumulative disease index of rasagiline at 3 mg/kg in the chronic phase. Monitoring of the chronic phase was performed upto day 51 post-immunization; 28 days after last dose. Black arrows indicate the time of the beginning of the administration of the compounds. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using ANOVA. (**H**) The effects of ORY-2001 at 0.5 mg/kg and ORY-LSD1 at 0.18 mg/kg on the mean clinical score and (**I**) on the cumulative disease index in the sub-chronic phase. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using ANOVA.

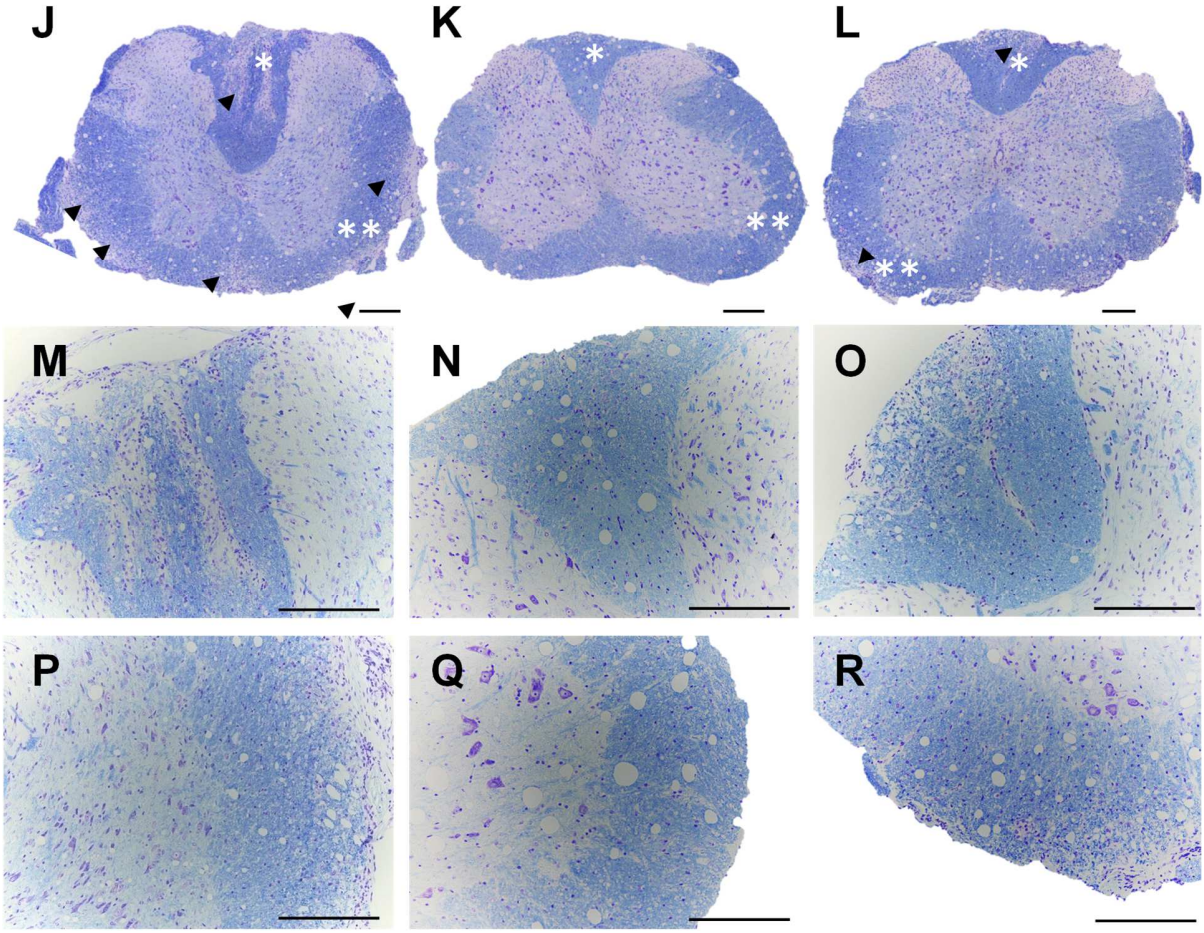

Figure A1 (continued). Therapeutic effects of ORY-2001 in EAE mice

Infiltration of inflammatory cells and demyelination as seen in Kluver-Barrera stained cervical section of spinal cords isolated from EAE mice after two weeks of treatment with (**J**) Vehicle, (**K**) 0.5 mg/kg ORY-2001 or (**L**) 0.18 mg/kg ORY-LSD1; and the corresponding magnified areas (**M**,**P**), (**N**,**Q**) and (**O**,**R**) labeled with (*,**). Arrowheads point to areas of demyelination and inflammatory cell infiltration. Scale bar = 200 µm.

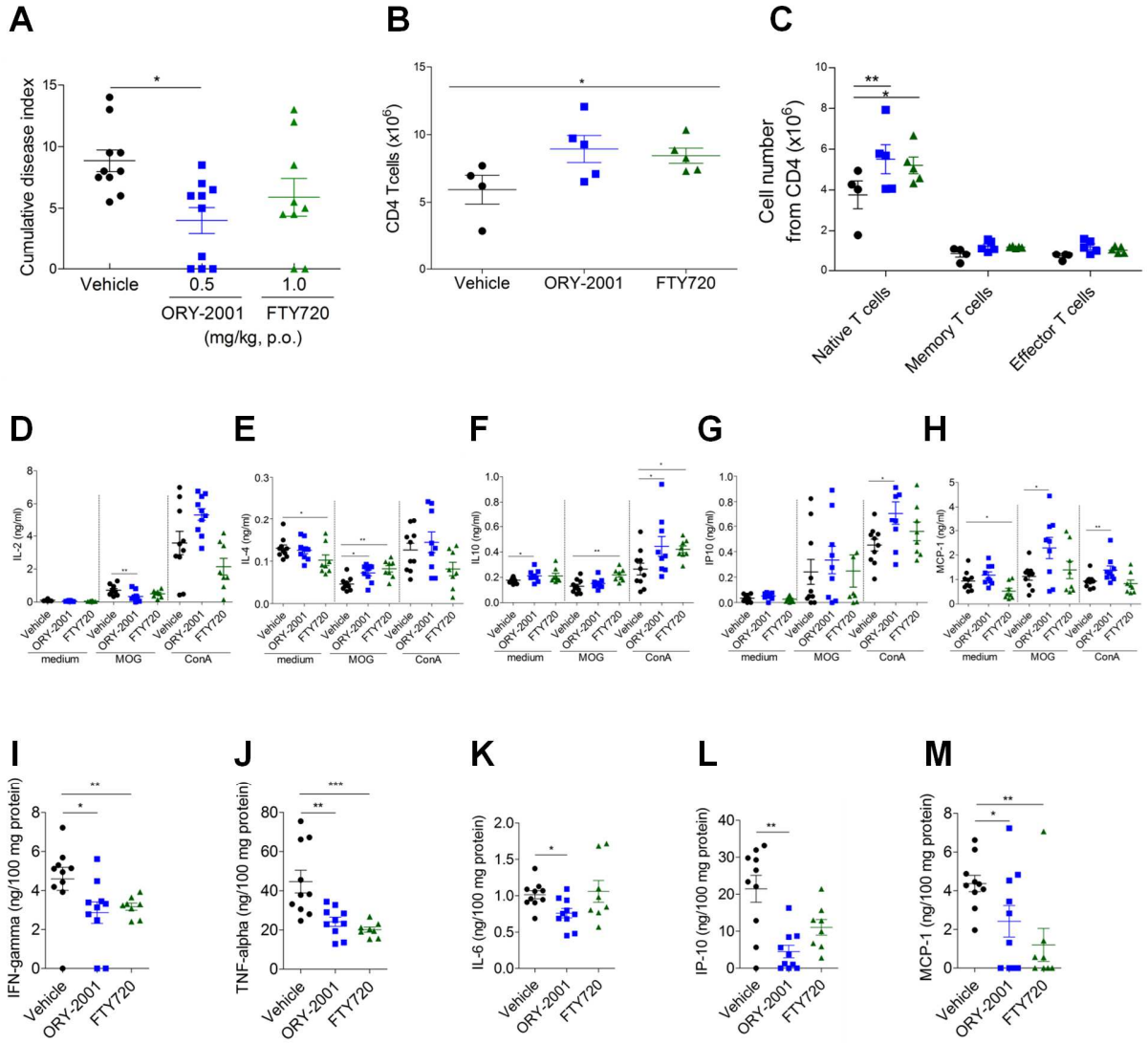

Figure A2. **Effect of treatment with ORY-2001 and FTY720 on EAE mice in the effector phase**

(**A)** Cumulative disease index of EAE mice treated with Vehicle ● (black), ORY-2001 ■ (blue) at 0.5 mg/kg and FTY720 ▲(green) at 1.0 mg/kg after 5 days of treatment, in the effector phase. (**B-C**) Cells isolated at EAE peak were assayed for T cell lineage expression by flow cytometry in the CD4 population (CD4CD44low/CD62Lhigh as naïve T cells, CD4/CD44high/CD62Llow as memory T cells and CD4/CD44medium/CD62Llow as effector T cells). N= 4-5 mice / group. Treatment with Vehicle ● (black), ■ (blue) ORY-2001 (0.5 mg/kg) and ▲ (green) FTY720 (1.0 mg/kg). Effect of culture in medium with or without MOG or ConA on the release of (**D**) IL2 from spleen cells, and of (**E**-**F)** cytokines and (**F**-**G**) chemokines from lymph node cells. Cells were obtained from N = 8-10 mice / group. (**I**-**L**) Effects of treatment on the release of cytokines and chemokines in the mouse brain. N= 8-10 mice / group. Statistical differences were indicated as vehicle vs ORY-2001 or FTY720; *p < 0.05; **p < 0.01; ***p < 0.001 using ANOVA followed by Tukey's multiple comparison test or Mann-Whitney test.

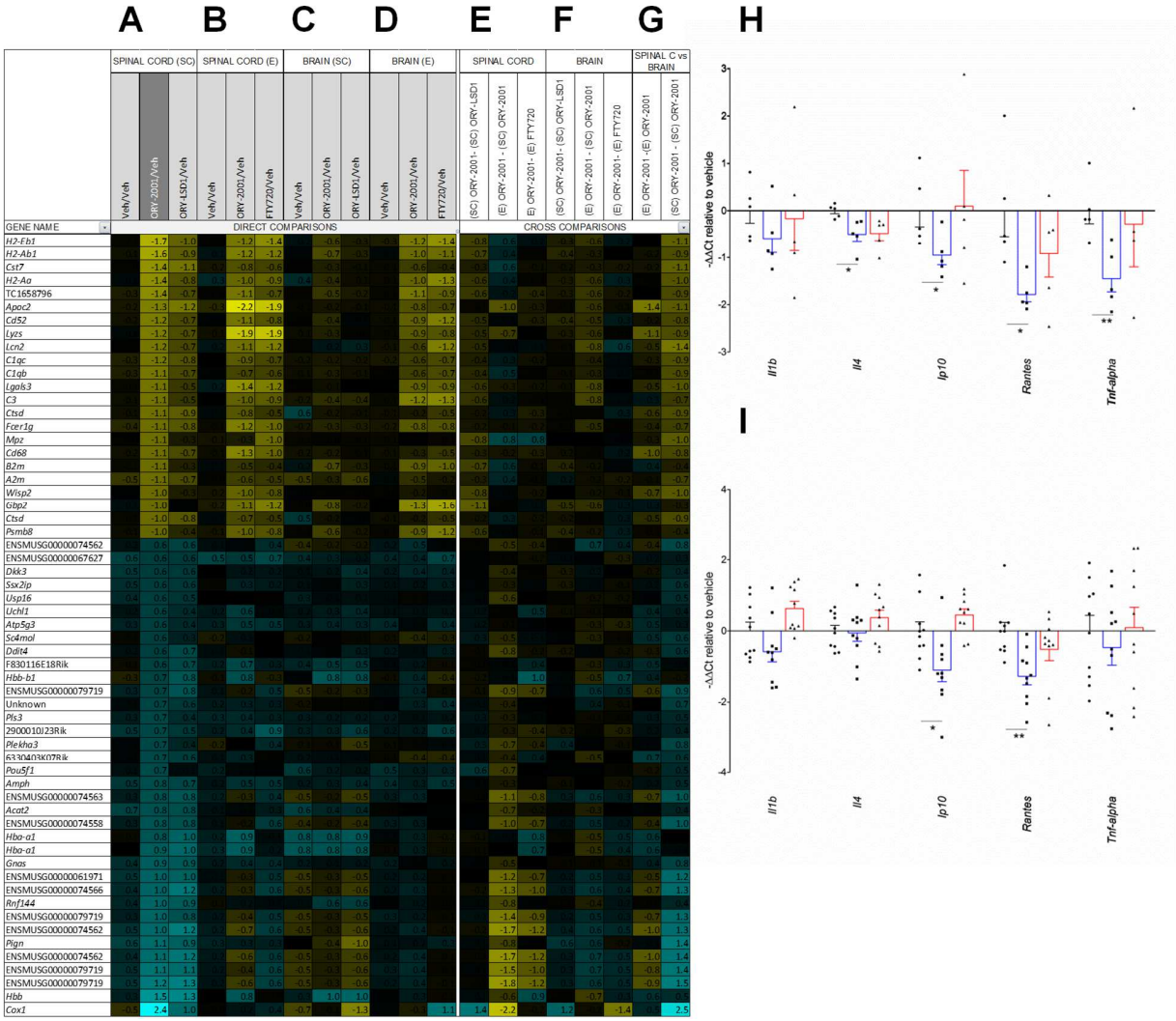

Figure A3. Gene expression analysis of ORY-2001, ORY-LSD1, FTY720

(**A**) Microarray survey of gene expression changes in the spinal cord in the sub-chronic phase; 3 days after the last dose. Animals were treated with Vehicle, ORY-2001 at 0.5 mg/kg or ORY-LSD1 at 0.18 mg/kg. (**B**) Microarray survey of gene expression changes in spinal cord in the effector phase, after treatment. Animals were treated with Vehicle, ORY-2001 at 0.5 mg/kg or FTY720 at 1 mg/kg. (**C**) Microarray survey of gene expression changes in brain in the sub-chronic phase; 3 days after the last dose. Animals were treated with Vehicle, ORY-2001 at 0.5 mg/kg or ORY-LSD1 at 0.18 mg/kg. (**D**) Microarray survey of gene expression changes in spinal cord in the effector phase after treatment. Animals were treated with Vehicle, ORY-2001 at 0.5 mg/kg or FTY720 at 1 mg/kg. (**E**) Comparison of the effect of the different treatments and disease phases in the spinal cord. (**F**) Comparison of the effect of the different treatments and disease phases in the brain. (**G**) Comparison of the effects of ORY-2001 in spinal cord and brain, in both phases. Pooled RNA from N = 5 spinal cords or 10 brains of vehicle, ORY-2001 or ORY-LSD1 treated mice or from N = 4 spinal cords or 6 brains of FTY720 treated mice was used to perform each survey, n = 3 replicate probes within an array. Gene expression changes are expressed as Log_2(Treatment/Veh) for direct comparisons, and as the difference of the Log_2(Treatment/Veh) values for the respective conditions in the cross-comparisons. Genes up-regulated > 1.5 fold (Log_2(ORY-

2001/Veh) > 0.6) or down-regulated > 2 fold (Log_2(ORY-2001/Veh) < -1) by ORY-2001 in the spinal cord in the sub-chronic phase were selected and represented for all comparisons. (**H**-**I**) qRT-PCR validations of selected individual genes modulated by ORY-2001 (blue), and ORY-LSD1 (red) in the sub-chronic phase in **H**) spinal cord and **I**) brain. Data are represented as -ΔΔCt values and mean ± SEM (n = 5 - 10 mice/group). Statistical analysis between Veh and ORY-2001 was calculated using the t-test. Welch's t-test was applied when the populations had unequal variances. * $p < 0.05$; ** $p < 0.01$.