Evobrutinib promotes myelin regeneration in two different models of demyelination*

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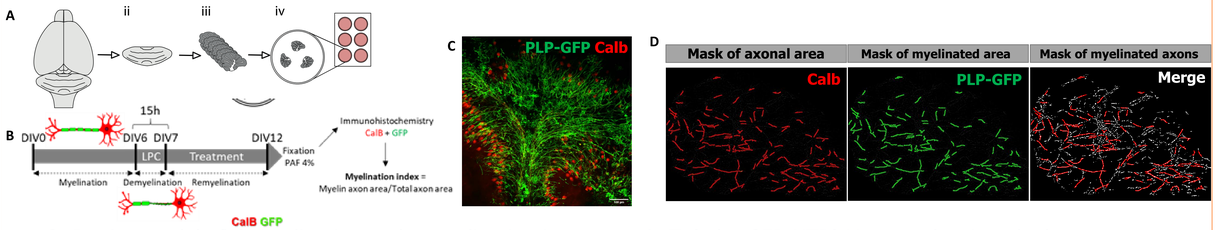
INTRODUCTION

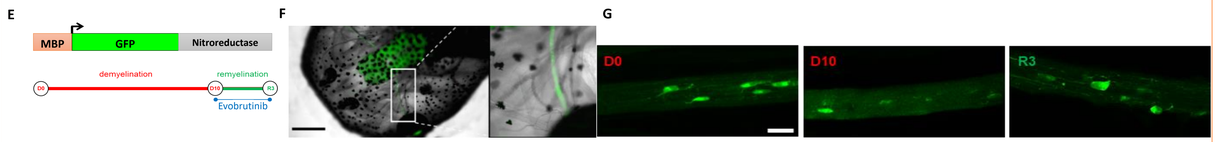
Microglia are the resident macrophages of the central nervous system (CNS). In multiple sclerosis (MS) and related experimental models, microglia have either a pro-inflammatory or a pro-regenerative/pro-remyelinating function. Inhibition of Bruton's tyrosine kinase (BTK), a member of the Tec family of kinases, has been shown to block differentiation of pro-inflammatory macrophages in response to granulocyte–macrophage colony-stimulating factor *in vitro*. However, the role of BTK in the CNS was unknown. We have previously shown that BTK is expressed by microglia and that an inhibitor of BTK (BTKi) promoted remyelination both in mouse and xenopus (Martin et al., *Brain Plasticity* 2020). Here, we investigated the effect of Evobrutinib, (a BTKi in phase III clinical trial) on remyelination. We compared the effect of Evobrutinib (EvoB) and Clemastine (Clem) on remyelination and on microglial cells recruitment during remyelination both *ex-vivo* on cerebellar slices of a transgenic mouse Tg*(plp:GFP),* to monitor the remyelination process after lysolecithin-induced demyelination, and *in vivo* in a conditional demyelination transgenic model developed in *Xenopus laevis* (Tg(*mbp:GFP-NTR)* well adapted to screen pro-remyelinating compounds.

METHODS

1- Ex vivo model of demyelination and remyelination in organotypic culture of mouse Tg(plp:GFP) cerebellum slices



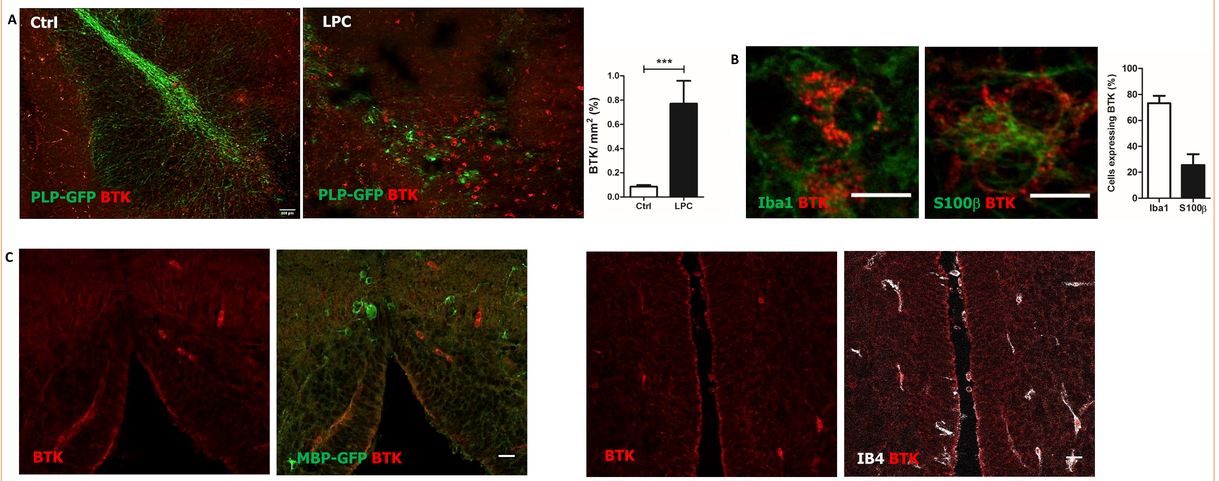
2- In vivo model of demyelination and remyelination in transgenic Tg(mbp:GFP-NTR) Xenopus laevis tadpoles



A) Protocol of organotypic slice culture generation from Tg(*plp:GFP)* mouse at P9, (Thetiot et al., Jove 2019). **B)** After 6 days in culture, demyelination was induced by addition of lysophosphatidylcholine (LPC) to the medium for 16–17 h. Then slices were transferred back to normal medium. At DIV9 (peak of demyelination), Evobrutinib and/or Clemastine were added for 4 days. Slices were fixed before immunostaining. **C)** Immunostainings of Calb on Tg(*plp:GFP)* mouse cerebellar slices. **D)** Myelination index was calculated semi-automatically using a macro developed on ImageJ, (Baudouin et al., 2021 ; Ronzano et al., 2021) from the quotient of the mask for axonal area (Calbindin signal) over a mask for myelinated axonal area (PLP signal overlapping with Calbindin signal). **E)** Tg(*mbp:GFP-NTR) Xenopus*, expresses GFP reporter fused to *E. coli* nitroreductase (NTR) under the control of mouse 1.9kb myelin basic protein regulatory sequence (*mbp).* Demyelination was induced by adding metronidazole (MTZ), a substrate of NTR enzyme, in the swimming water. **F)** In MTZ-exposed transgenic Tg(*mbp:GFP-NTR)* tadpoles demyelination is restricted to the CNS and effect of molecules was analyzed in live animals by counting the number of GFP positive cells in the optic nerve. **G)** At D10, at the end of MTZ exposure Evobrutinib and/or Clemastine was added in swimming water for 3 days (R3) and renewed once. Quantification of spontaneous remyelination (control) was compared to remyelination in the presence of Evobrutinib and/or Clemastine at R3.

RESULTS

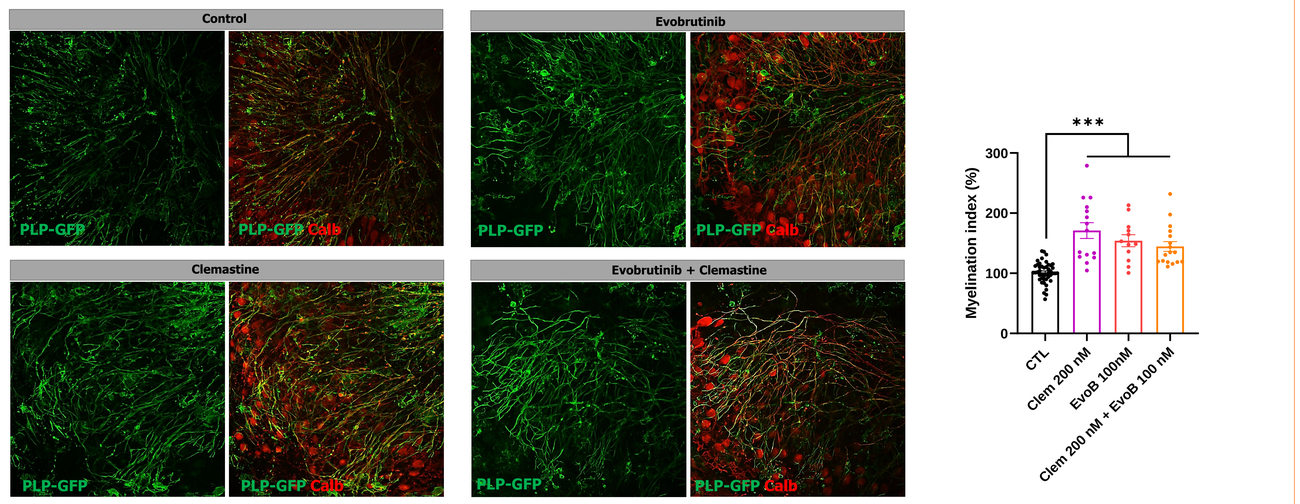
1- Expression of BTK in microglia cells upon demyelination



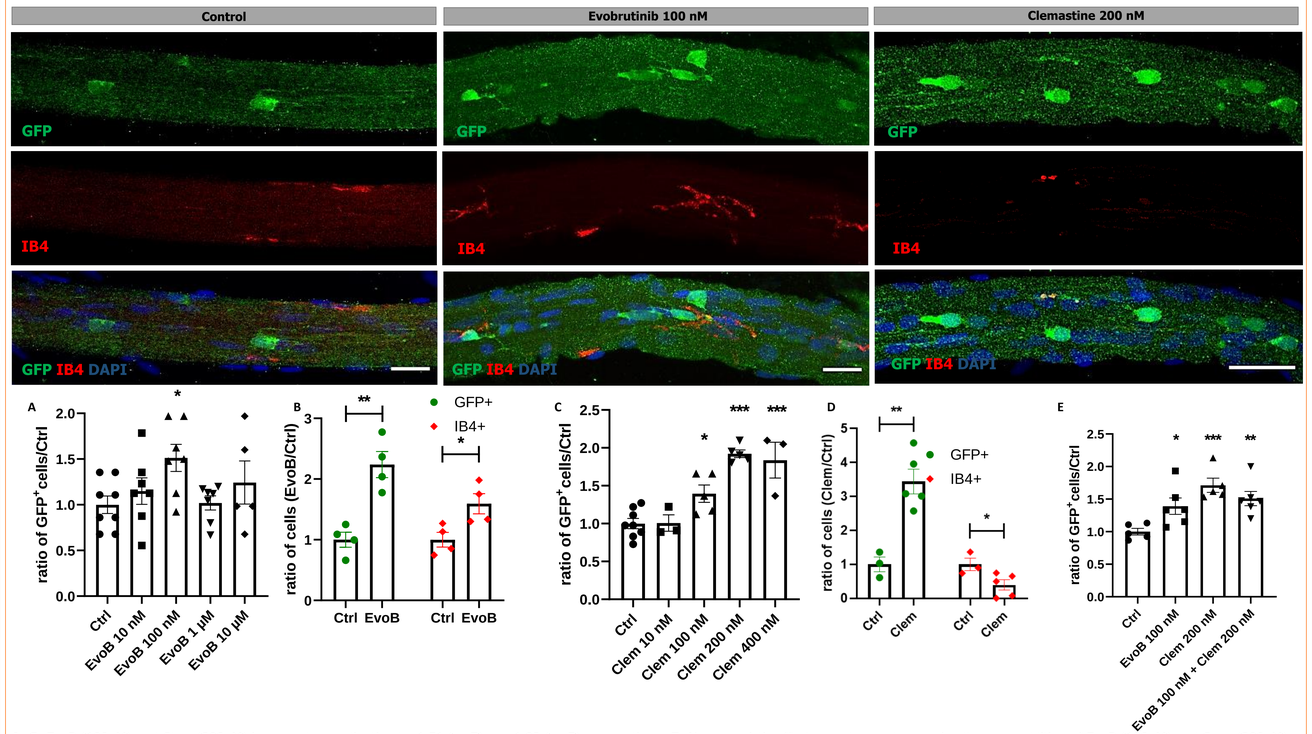
A) Immunostainings for BTK (red) on cerebellar organotypic slices from P9 Tg(*plp:GFP)* mouse. LPC treatment induced a demyelination (loss of GFP signal) and a concomitant 8.5-fold increase in BTK signal. **B)** immunostaining was performed for BTK (Red) and Iba1 (microglia, green) or S100b (astrocyte, green). **C)** Immunodetection of BTK in microglial cells, but not in oligodendrocytes. Coronal or horizontal tissue sections across the brain stem off stage 52-53 Tg(*mbp:GFP-NTR)* tadpoles doubly labeled with anti-BTK (red) and anti-GFP (oligodendrocytes, green) antibodies or isolectin IB4 (microglia, white). Scale bar: A: 100µm B: 10µm C: 20µm.

RESULTS

2- Remyelination in cerebellar organotypic slices after Evobrutinib and/or Clemastine treatment



3- Dose response of Evobrutinib and/or Clemastine on remyelination and microglial recruitment *in vivo*



A, C: EvoB (100nM) and Clem (200nM) increased remyelination by 1.51 (n=7) and 1.92 (n=5) , respectively. **E:** No synergistic effect was observed upon simultaneous addition of EvoB (100nM) and Clem (200nM). **B:** EvoB (100nM) increased the number of GFP+ and IB4+ cells by 2.24 and 1.59(n=4), respectively. **D:** Clem (200nM) increased the number of GFP+ cells by 3.31, but decreased the number of IB4+ cells by 0.48 (n=5).

CONCLUSIONS

Using two different and complementary experimental models of demyelination: *ex vivo* LPC-treated mouse cerebellar organotypic slices and *in vivo* conditionally demyelinated Xenopus tadpole, we demonstrated that Evobrutinib, similarly to Clemastine favored remyelination. We also showed an absence of synergistic effect of the two molecules. Evobrutinib induced recruitment of both oligodendrocytes and microglial cells, while Clemastine enhanced the number of GFP+ cells, but decreased the number of microglia/macrophages demonstrating two different mechanisms of action to promote remyelination. Altogether, our data show that BTK inhibition improved myelin regeneration suggesting that BTK inhibition represents a promising new therapeutic strategy to promote remyelination by targeting microglia.

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