Role of Neuropilin-1 Receptor in Controlling Cortical Inter-Hemispheric Circuits

F. Martín-Fernández[1], C.G. Briz[1], M. Nieto[1]

[1]Centro Nacional de Biotecnología (CNB-CSIC), Campus de Cantoblanco, C/ Darwin 3, 28049, Madrid, Spain

The mechanisms that regulate the formation of inter-hemispheric circuits are only partially understood. During development, multiple cues expressed in the brain are crucial for the wiring of stereotyped interhemispheric maps. Neuropilin-1 (Nrp1) and its ligands are thought implicated in controlling the selection of the dorsal-ventral callosal pathways leading to contralateral targets. To understand additional roles of Nrp1, we combined in utero electroporation experiments with retrograde tracer injections in C57BL6'J mice. We over-expressed and knocked-down Nrp1 in subpopulations of layer 2/3 neurons of the primary (S1) and secondary (S2) somatosensory cortex. Then, we analyzed the number and distribution of callosaly projecting neurons (CPN) labeled by injections of fluorescently conjugated cholera toxin subunit B (CTB) into the corpus callosum (CC) in the contralateral hemisphere at postnatal day (P) 16 and P30. Brains were histologically processed and confocal images were taken to analyze the contralateral axonal pattern and to quantify the percentage of CPN in the electroporated population as well as in non-electroporated cells. The results show that CPN with somas located in S2 are sensitive to changes in Nrp1 levels whereas no changes were detected in the number of S1 CPN or its branches. Knocking-down Nrp1 reduced S2 CPN in numbers; it also reduced the amount of axonal branching in the contralateral S2 territories. Nrp1 over-expression also reduced S2 innervation but did not alter the number or distribution of CPN. Our results suggest that during CC formation, Nrp1 regulates post-crossing events and not only the dorsal-ventral midline CC trajectories. Ongoing studies are intended to confirm the hypothesis that Nrp1 levels control the axonal selection of targets in interhemispheric circuits.

Experimental Approach

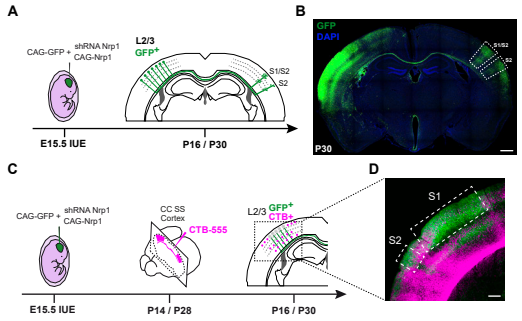

Figure 1: **Experimental approach. A** *In utero* electroporation (IUE) at embryonic day (E) 15.5 targeting layer 2/3 neurons (L2/3) with GFP and shRNA Nrp1 (to knock-down) and CAG-Nrp1 (to overexpress). **B** Coronal section of electroporated L2/3 neurons with GFP (control) in somatosensory (SS) cortex at postnatal day (P) 30. GFP+ neurons (ipsilateral side) extend their axons through the CC and cross the midline to the other hemisphere (contralateral side) where they form two axonal columns (S1/S2 and S2) (Scale bar = 500 µm). **C** IUE followed by stereotaxic injections at CC in SS cortex with retrograde tracer cholera toxin subunit B (CTB) at P14 or P28. Brains were processed at P16 or P30. **D** Detail of coronal section of P30 control brain in ipsilateral SS cortex. In magenta, callosal projection neurons (CPN). In green, electroporated L2/3 neurons (Scale bar = 200 µm).

Contralateral Innervation Pattern in knock-down and over-expression of Nrp1

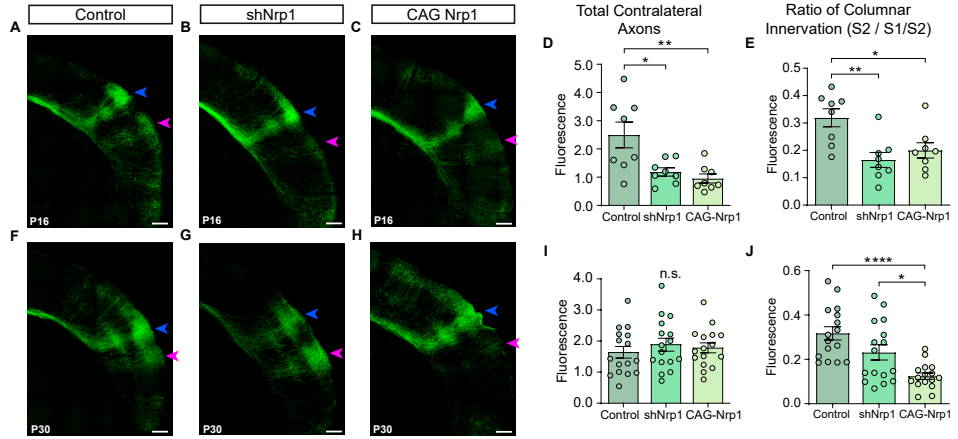

Figure 2: **Innervation pattern of L2/3 SS CPN at P16 and P30. A-C** Contralateral pattern at P16 in control, shNrp1 and CAG Nrp1. Blue arrow: S1/S2 column. Pink arrow: S2 column. **D** Quantification of total contralateral axons at P16. Both experimental conditions present a decrease in total contralateral innervation at P16. **E** Axonal ratio between both columns at P16. Both conditions present a decrease in S2 column innervation. **F-H** Contralateral pattern at P30 in control, shNrp1 and CAG Nrp1. **I** Quantification of total contralateral axons at P30, where a few non-significant increase is observed in total innervation. **J** Axonal ratio between both columns with a similar decrease in S2 axons presence. (One-way ANOVA. *** p-value < 0.0001 ** p-value < 0.005; * p-value < 0.05. (Scale bars: 300 µm).

Nº of CPNs in electroporated population at P16

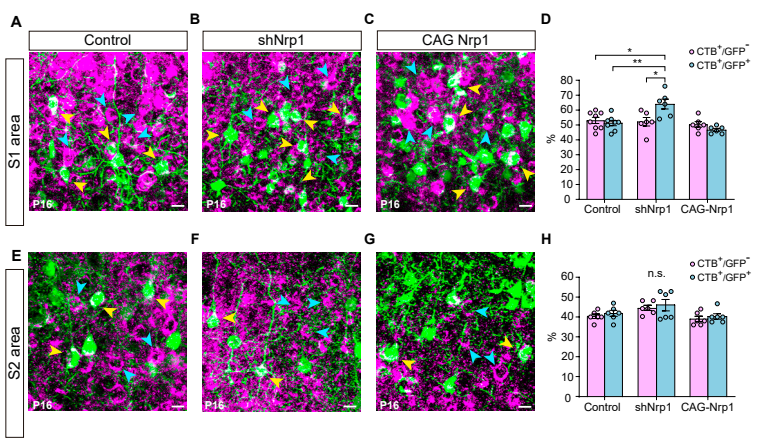

Figure 3: callosal proportions of electroporated neurons in S1 and S2 at P16. **A-C** Detailed images of electroporated callosal neurons in control, shNrp1 and CAG Nrp1 in S1 area at P16. **D** Quantification of the percentage of CTB+ neurons over GFP- population (pink) and CTB+ neurons over GFP+ population (blue) in S1L2/3. At this stage, knocked-down Nrp1 induces an increase of 15% of callosity compared to control. **E-G** Detailed images of electroporated callosal neurons in control, shNrp1 and CAG Nrp1 in S2 area at P16. **H** Quantification of the percentage of CTB+ neurons over GFP- population (pink) and CTB+ neurons over GFP+ population (blue) in S2L2/3 (Yellow arrows: electroporated callosal neurons(GFP+CTB+). Blue arrows: non-electroporated callosal neurons (GFP-CTB+). Two-way ANOVA: * p-value < 0.05, ** p-value < 0.001. Scale bars = 25 µm).

Nº of CPNs in electroporated population at P30

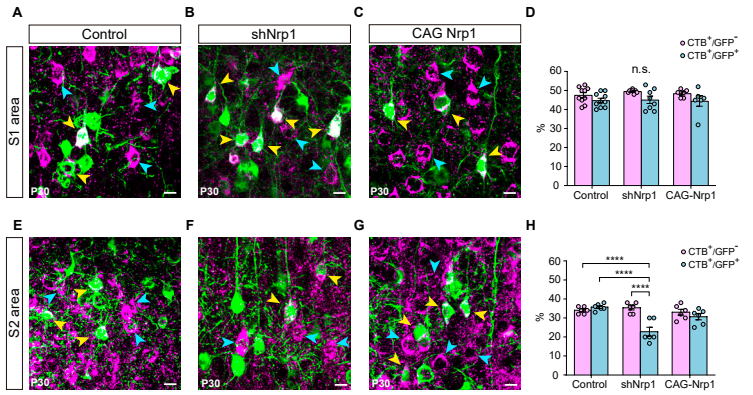

Figure 4: callosal proportions of electroporated neurons in S1 and S2 at P30. **A-C** Detailed images of electroporated callosal neurons in control, shNrp1 and CAG Nrp1 in S1 area at P30. **D** Quantification of the percentage of CTB+ neurons over GFP- population (pink) and CTB+ neurons over GFP+ population (blue) in S1L2/3. **E-G** Detailed images of electroporated callosal neurons in control, shNrp1 and CAG Nrp1 in S2 area at P30. **H** Quantification of the percentage of CTB+ neurons over GFP- population (pink) and CTB+ neurons over GFP+ population (blue) in S2L2/3 (Yellow arrows: electroporated callosal neurons(GFP+CTB+). Blue arrows: non-electroporated callosal neurons (GFP-CTB+). Two-way ANOVA: **** p-value < 0.0001. Scale bars = 25 µm).

Nrp1 controls homotopic callosity

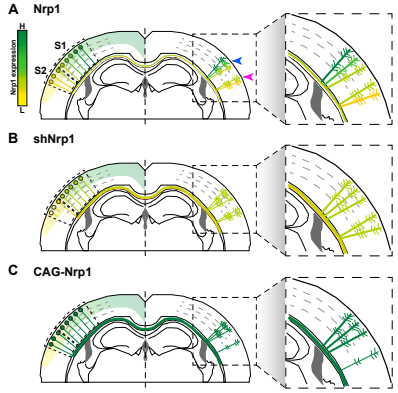

Figure 5: **A** Nrp1 is expressed in the cortex in a high-medial, to low-lateral, gradient (higher levels in green, lower levels in yellow). Accordingly, S1L2/3 neurons (green dots) express high to intermediate levels of Nrp1. S2L2/3 neurons express low levels (light green and yellow dots). L2/3 neurons expressing high to intermediate levels of Nrp1 branch preferentially in homotopic S1/S2 column (blue arrow), and S2L2/3 neurons expressing intermediate to low levels into S2 areas (magenta arrow). **B** Knocking down Nrp1 reduces the levels of Nrp1 according to the gradient. L2/3 CPNs with intermediate levels of Nrp1 expression can branch both in S1/S2 and S2. As consequence, an exceeding number of heterotopic branches from S1L2/3 CPNs outgrowth ectopically in the S2 column. They may outcompete axons of shNrp1 targeted S2L2/3 neurons that express very low levels of Nrp1 (light yellow dots). Many of these S2L2/3 neurons cannot terminate innervation and refine their callosal axon during the late period of P16-P30 developmental CPN refinement, thus becoming ipsilateral-only connecting neurons. **C** Neurons over-expressing Nrp1 branch in the S1/S2 column but are not competent to innervate S2 areas, which is then significantly reduced.

Conclusions

Neuropilin-1 regulates post-crossing events and the refinement of callosal axons in L2/3 neurons:

- Nrp1 over-expression and knock-down produce a significant loss of axons in the S2 column at P16 and P30. Knocking-down Nrp1 induce less contralateral axonal presence due to a reduction in S2 CPNs. However, over-expression of Nrp1 decreases axonal branching without affecting CPNs proportions.

- During development (P16), the innervation pattern is similar to P30, although shNrp1 induces more callosal neurons in the S1 area. Both experimental conditions produce a reduction of total contralateral axons, maybe due to a retarded stabilization of callosal axons in the cortical plate.

- Nrp1 expression levels control homotopic innervation of callosal axons. When knocking-down Nrp1, more lateral CPNs are more affected and are unabled to maintain their homotopic projections. Over-expression of Nrp1 induces a scarce branching of more lateral S2 CPNs, but does not affect their callosity.

References

1. Martín-Fernández, F., et al. (2021). Role of Nrp1 in controlling cortical interhemispheric circuits . eLife (*under revision*).

2. Matsuda, T., and Cepko, C.L. (2007). Controlled expression of transgenes introduced by in vivo electroporation. Proc. Natl. Acad. Sci. USA 104, 1027–1032.

3. Price, D.J. and Innocenti, G. M. (2005). Exuberance in the development of cortical networks. Nat. Rev. Neurosci. 6, 955-965.

4. De Leon-Reyes, N.S. et al. (2019). Transient callosal projections of L4 neurons are eliminated before the acquisition of local connectivity. Nat. Comm.

5. Wu, K. Y., M. He, et al. (2014). Semaphorin 3A activates the guanosine triphosphatase Rab5 to promote growth cone collapse and organize callosal axon projections. Sci Signal.

Acknowledgments: This work was funded by grants from MINECO (SAF-2017-83117-R) and ERA-Net Neuron (PCIN-2015-176-C02-02). F.M.F holds a FPU (FPU15/02111) fellowship from the Spanish "Ministerio de Educación y Formación Profesional".

Contact: mnlopez@cnb.csic.es / fmartin@cnb.csic.es