Figure 1

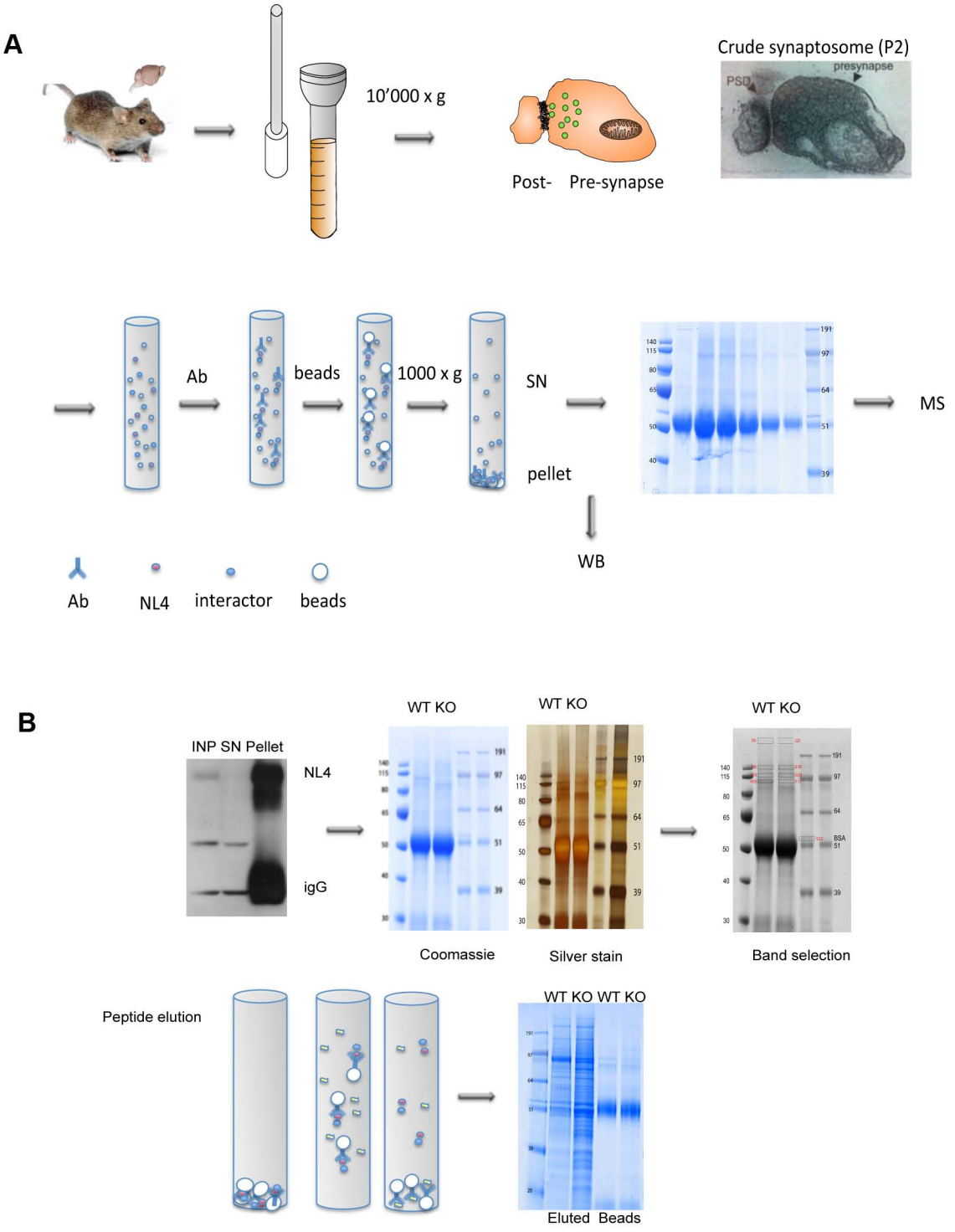

Figure 1: Optimised protocol for immunoprecipiataon of transmembrane proteins from whole brain. (A) Two 6-8 Wk mouse brains were pooled and homogenised from either WT or NL4 KO animals per treatment. Following centrifugation protocols, the P2 crude synaptosome fraction was obtained. The P2 was then resuspended in detergent at 2 mg/ml and insoluble material removerd by ultracentrifugation. IP was carried out following preclearance of the supernatant with 50 µl bed Vol of protein A sepharose for 30 min. Anti-NL4 antibody (5 µl per brain) was added and incubated for 2 h at 4 °C, followed by sepharose A as previous for a further 2 h. The pellet was washed with ACSF with detergent and resuspended in 1X Laemlli buffer for MS analysis or further processed for peptide elution. (B) In order to remove IgG and non specific binders to the beads from the MS sample, a peptide elution step was introduced. The beads were incubated at 37 °C with ACSF containing an excess of NL4 peptide (0.5 mg/ml). This procedure sucessfully eluted NL4 into the supernatant leaving heavy and light IgG chains on the beads.

Figure 2

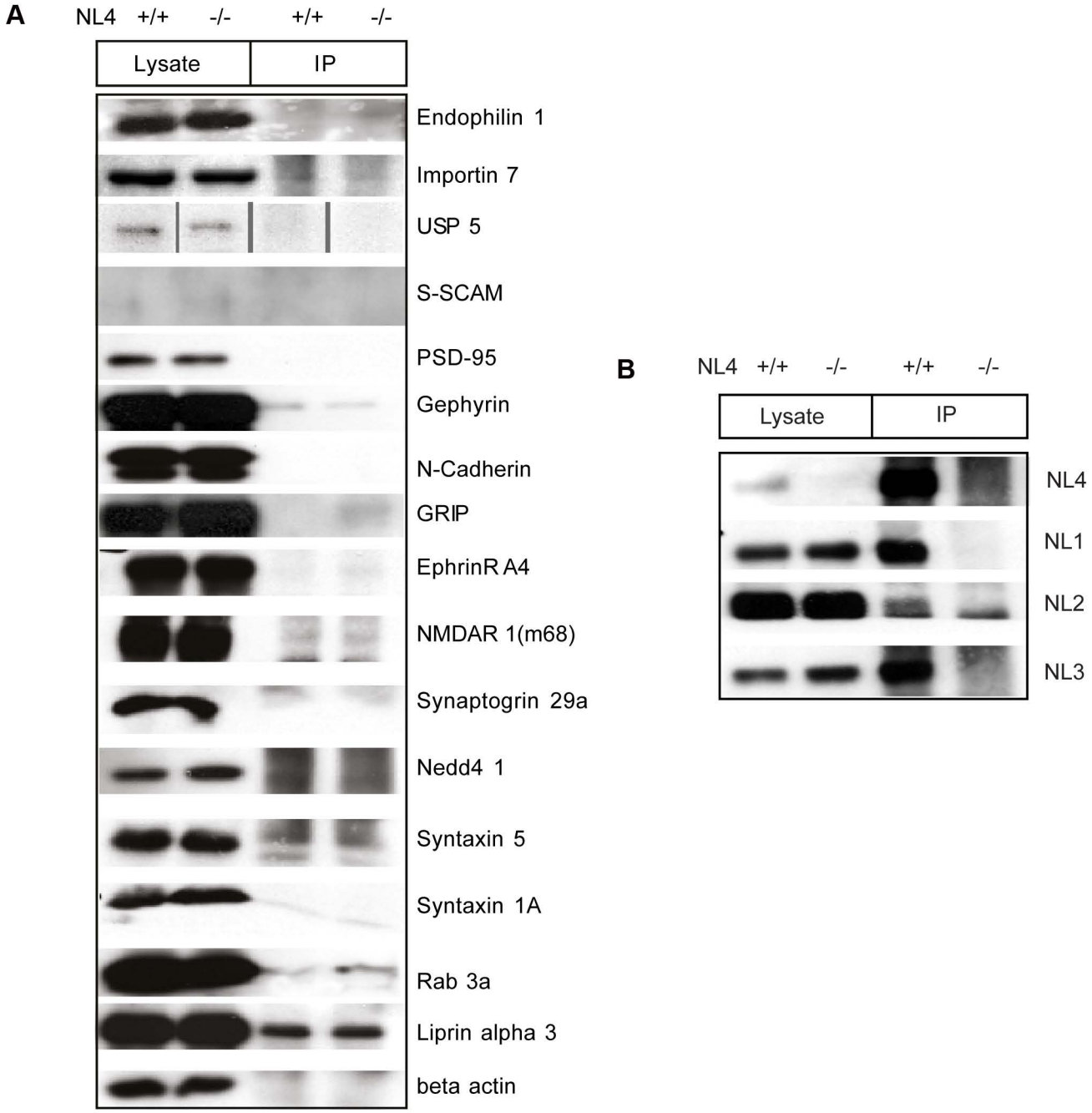

Figure 2: Validation of MS binding partners by Western blotting (A) Multiple proteins that resulted in 1/2 peptides being detected by MS were tested via Western blotting for their presence in WT but not NL4 KO IP samples. Proteins were detectable in lysates but not IP samples or could be detected in both IP-samples. None of the tested proteins therefore proved to be valid interactors. (B) All other neuroligin iso-forms however were detected by Western blotting in WT but not in NL4 KO IP samples following IP for NL4.

Figure 3

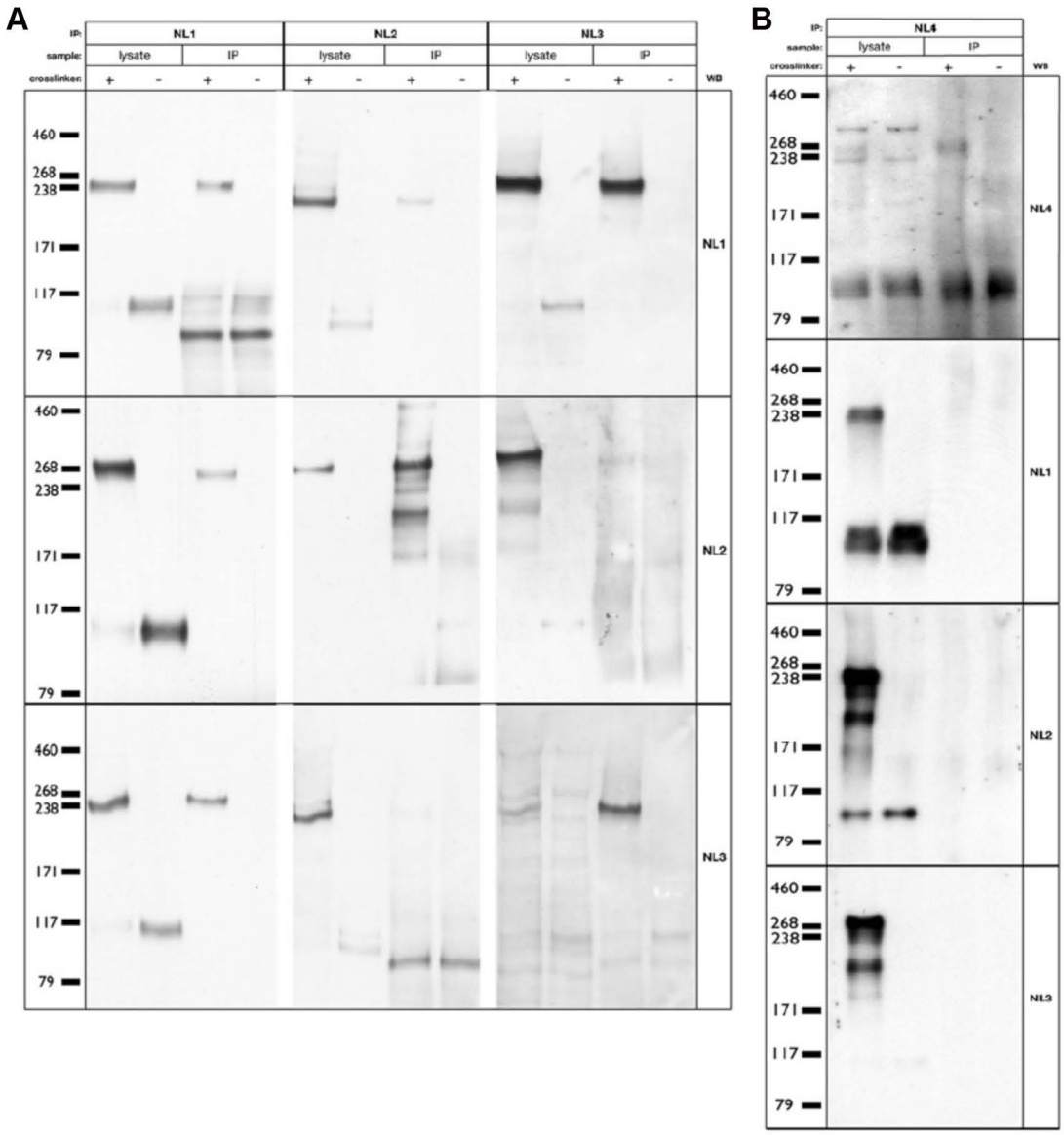

Figure 3: Neuroligins form specific heterodimer species (Poulopoulos, Soykan, 2012). (A) Cross-linking and imunoprecipitation experiments showing Western blotting of NL1, NL2 and NL3 from cross-linker-treated (+) or mock treated (−) hippocampal neuron lysates (lysate) and fractions immunopurified (IP) for NL1, NL2 or NL3. Each row depicts blots of one neuroligin isoform as indicated on the right-hand side. Load is 2 % of total lysate, and 20 % of the total fractions immunopurified. Cross-reacting bands appear in immunopurified lanes at 90 kDa when precipitating and blotting antibodies are of the same isotype. (B) Equivalent analysis as in (A), with Western blotting of all four of the neuroligin isoforms from hippocampal neuron samples immunopurified for NL4. Analysis of endogenous isoform pairs indicates abundant NL1–NL3, and minor NL1–NL2 heterodimers, but no reproducibly detectable NL2–NL3 or NL4-containing heterodimers in the cultures examined. Electrophoretic mobility of protein marker standards are given in kDa on the left-hand side of the blots.

Figure 4

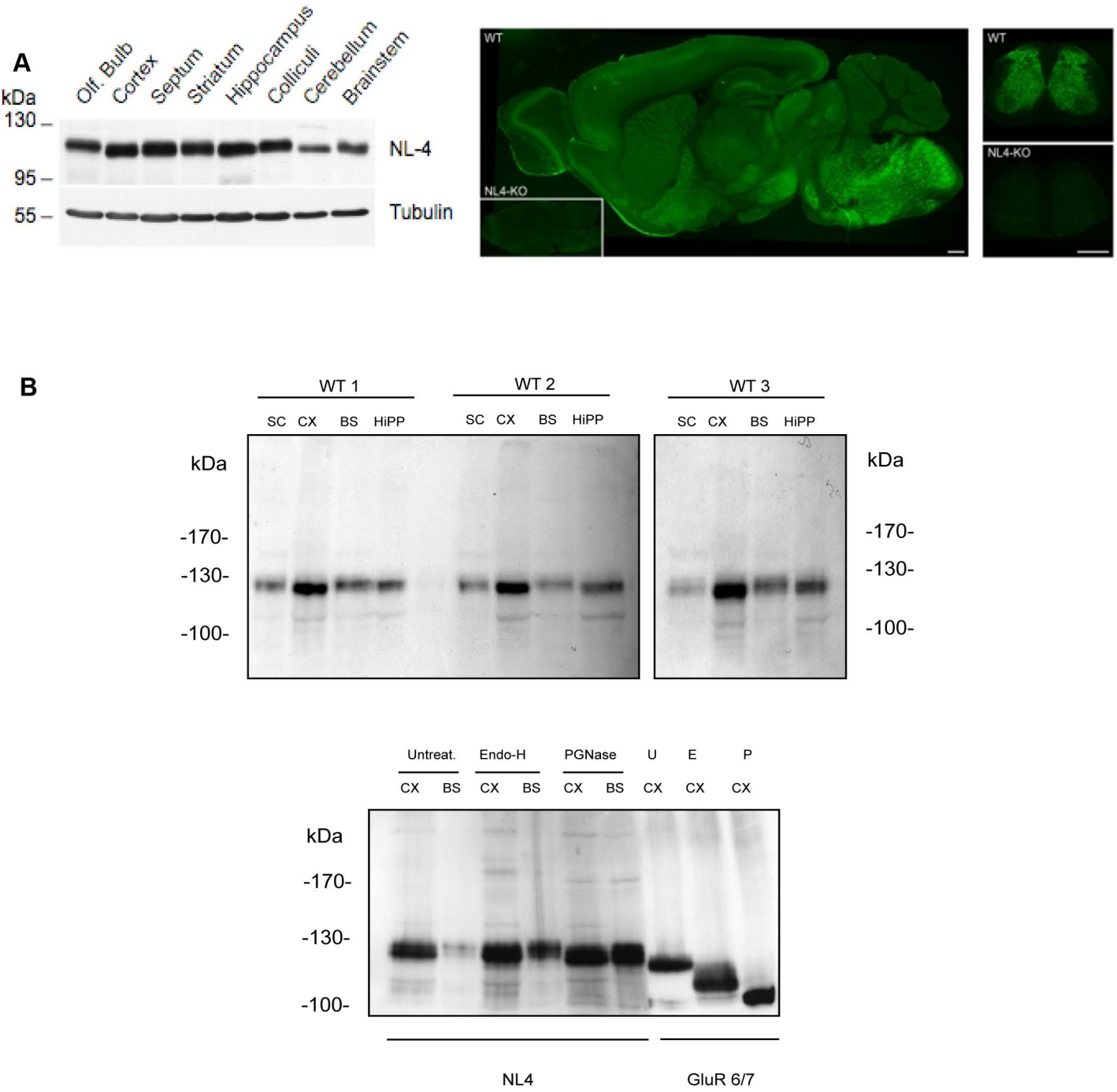

Figure 4: Neuroligin 4 may possess a brain region specific differential conformation or expression pattern. (A) Western blotting (left) and immunohistochemical staining of brain and spinal cord (right) of NL4. Expression levels of NL4 did not correlate betwen the staining and blotting, despite both signals being specific, as can be observed by the absence of signal in NL4 KO. Scale bar= 500 µm (B) NL4 migrates at slightly different sizes in different brain regions (top), which may indicate differential post-translational modification. Treatment with PNGase F (removing N-glycans) resulted in a collapse of this difference between the regions, indicating differential mature N-glycosylation. However pre-treatment of immunohistochemical stainings with PNGase F did not expose the antibody epitope allowing for specific stainings. No change was observed with Endo-H treatment. GluR 6/7 was used as a positive control for the enzymes and experimental protocol. SC- Spinal cord, HIPP-hippocampus, Cx-cortex, BS-brain stem, U, E, P short for untreated, Endo-H and PNGase treated respectively.