Palmitoylation of BMPR1a regulates neural stem cell fate

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Supplementary Materials and Methods

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Supplementary Figures

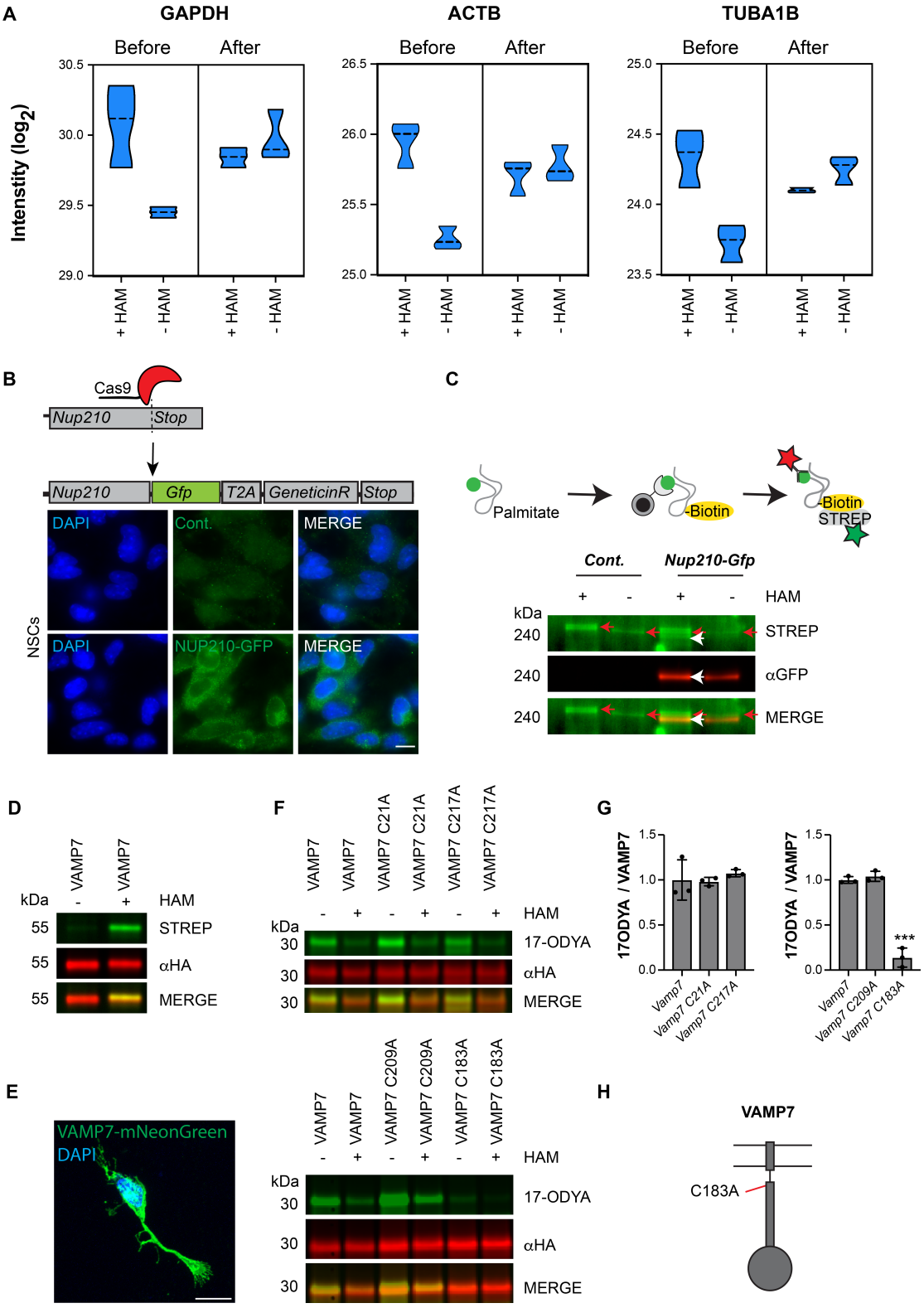
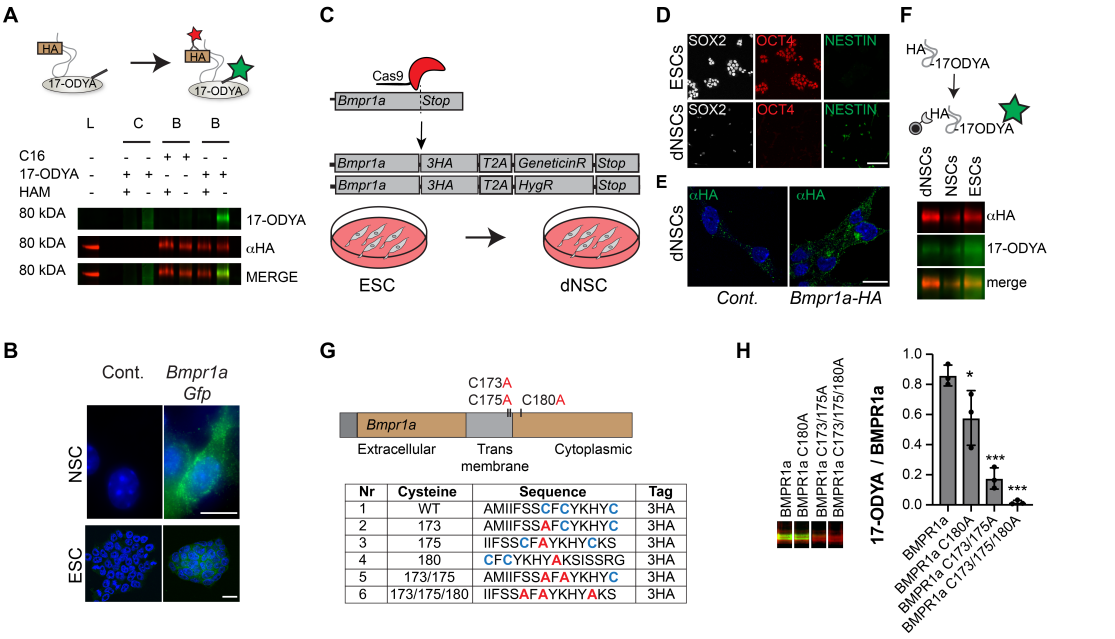


Fig. S1. Validation of S-acylated proteins. (A) The illustration shows the intensities for Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH), Beta-actin (ACTB), and Tubulin alpha-1B (TUBA1B) as measured by mass spectrometry before and after normalization (blue). Due to their high abundance these proteins bind to the beads in a palmitoylation-independent manner and therefore can be used as an internal control to normalize the intensities between different samples. (B) Upper panel: The illustration describes the CRISPR/Cas9 mediated tagging of endogenous *Nup210* with *Gfp*, followed by an ABE assay where the palmitate is exchanged for a biotin (yellow). Lower panel: Shown are pictures of control and NUP210-GFP tagged NSCs (green: anti-GFP, blue: DAPI). (C) Upper panel: The illustration describes an ABE assay where the palmitate is exchanged for a biotin (yellow). Lower panel: ABE analysis of untagged control cells and endogenously tagged NUP210 (red) confirms S-acylation (green, white arrowhead) of NUP210. The red arrowheads label unspecific bands. (D) ABE assay of Vesicle-associated membrane protein 7 tagged with mNeonGreen-HA (VAMP7-N-HA; red) overexpressed in adult NSCs confirms S-acylation (green) of VAMP7. (E) Shown is overexpressed VAMP7-N-HA (green) together with a DAPI counterstain (blue) showing that VAMP7 localizes to vesicles. (F-G) Metabolic labeling with 17-ODYA (alkyne-linked palmitate ortholog 17-octadecynoic acid) of overexpressed VAMP7 in NSCs shows that VAMP7 (red) is S-acylated (green) at cysteine 183. Mutation of the cysteines C21, C217 and C209 had no effect on the measured 17-ODYA intensities. It is therefore unlikely that those sites are palmitoylated. However, mutation of cysteine 183 to an alanine significantly decreases the 17-ODYA intensity. Thus, C183 was identified to be a palmitoylated site. (H) Schematic indicating that the S-acylated site at C183 within VAMP7 is localized close to the transmembrane domain. As palmitoylated residues can often be found in close proximity to the membrane this finding is in alignment with the localization of previously described palmitoylation sites. Error bars represent mean +/- S.D. Scale bars represent 10 µm (B,E), ***$p < 0.001$.



Nr	Cysteine	Sequence	Tag
1	WT	AMIIFSSCFCYKHYC	3HA
2	173	AMIIFSSAFCYKHYC	3HA
3	175	IIFSSCFAYKHYCKS	3HA
4	180	CFCYKHYAKSISSRG	3HA
5	173/175	AMIIFSSAFAYKHYC	3HA
6	173/175/180	IIFSSAFAYKHYAKS	3HA

Fig. S2. Validation of BMPR1a acylation in diverse cell types. (A) Metabolic labeling of overexpressed BMPR1a (red) with 17-ODYA (green) in NSCs reveals that the receptor is S-acylated. Note the intense band in green. L: ladder, C: control, B: BMPR1a overexpression, C16: palmitate, 17-ODYA (alkyne-linked palmitate ortholog 17-octadecynoic acid). (B) Endogenously tagged BMPR1a-GFP (green) localizes to the plasma membrane in NSCs and mouse ESCs. (C) BMPR1a was tagged in ESCs with a HA-tag and the ESCs differentiated into NSCs. Shown is an illustration highlighting the changes that were introduced into the genome. (D) Protein markers expressed change upon differentiation from ESCs to NSCs. ESCs: upper panel, SOX2+ (white), OCT4+ (red), NESTIN- (green); NSCs: lower panel, SOX2+ (white), OCT4- (red), NESTIN+ (green). (E) BMPR1a-HA localized to the plasma membrane in differentiated NSCs. Shown are immunofluorescence labelings against HA in ESC-derived control and *BMPR1a-HA* NSCs (green). Nuclei were counterstained using DAPI. (F) Metabolic labeling experiments using 17-ODYA reveal that BMPR1a (red) is S-acylated (green) in ESC-derived NSCs (first lane), NSCs isolated from the adult hippocampus (middle lane) and ESCs (last lane). (G) Shown is an illustration of the overexpression constructs that were used to confirm S-acylation of BMPR1a. S-acylated cysteines are shown in blue, cysteines that were mutated into alanines are shown in red. (H) Mutation of all the cysteines (C173, C175, C180) into alanines completely

abolishes S-acylation (green) of the BMPR1a (red). Scale bars represent 10 μm (A, NSC; E, dNSC), 20 μm (A, ESC), 100 μm (F). Error bars represent mean +/- S.D. *$p < 0.05$, ***$p < 0.001$

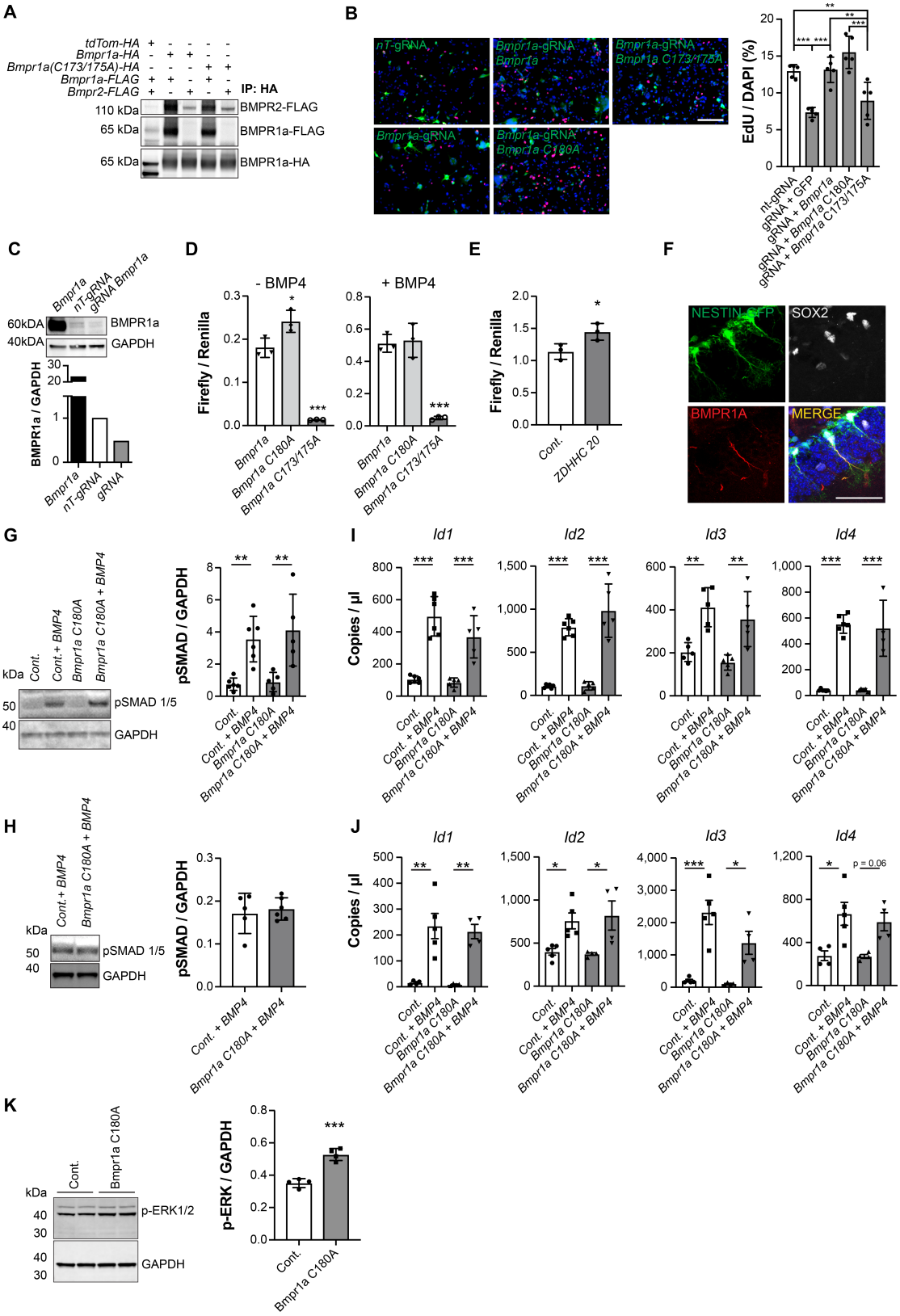


Fig. S3. S-acylation of cysteines 173 and 175 is critical for canonical BMP signaling. (A) BMPR1a C173/175A can interact with BMPR1a and

BMPR2. Shown are co-immunoprecipitations for HA-tagged BMPR1a and BMPR1a C173/175A overexpressed together with FLAG-tagged BMPR1a or BMPR2 in NSCs. HA-tagged tdTomato was used to control for unspecific binding. (B) CRISPR/Cas9 mediated knockout of BMPR1A shows that BMPR1A is important for proliferation of NSCs *in vitro.* Overexpression of gRNA resistant BMPR1a and BMPR1a C180A in BMPR1a knockout cells (grey) were able to restore proliferation levels to wild-type levels. Overexpression of resistant BMPR1a C173/175A was not able to significantly promote proliferation in BMPR1a knockout NSCs. (C) Validation of the gRNAs used to knockout *Bmpr1a*. Lane 1: BMPR1a overexpression (black), lane 2: nT-gRNA (white) lane 3: *Bmpr1a*-gRNAs (grey). GAPDH was used as loading control. (D) In contrary to BMPR1a C180A (light grey), overexpression of mutated BMPR1a C173A/C175A (grey) reduces canonical BMP signaling as measured by a BMP-responsive luciferase assay using *Id* gene regulatory elements in the absence and presence of the ligand BMP4. (E) Overexpression of zDHHC20 (grey) promotes canonical BMP signaling as measured by a BMP-responsive luciferase assay using *Id* gene regulatory elements. (F) Expression of BMPR1a (red) co-localizes with NESTIN+ (green) / SOX2+ (white) cells in the dentate gyrus of adult mice. Note the labeling of radial processes (red). (G) Stimulation with BMP4 activates canonical BMP signaling in control (white) and *Bmpr1a C180A* (grey) mutant NSC lines obtained from the dorsal ventricular zone of E17.5 embryos, as measured by P-SMAD-1/5 levels. (H) BMP4 promotes canonical BMP signaling, as measured by P-SMAD-1/5 levels in control (white) and mutant cell lines (grey) kept under differentiating conditions for 4 days. (I-J) Control (white) as well as *Bmpr1a C180A* (grey) cells are able to induce expression of *Id* genes upon stimulation with BMP4 under proliferative (I) as well as under differentiating (J) conditions as measured by digital droplet PCR. (K) Non-canonical BMP signaling is affected in differentiating *Bmpr1a C180A* cells (grey) compared to control cells (white), indicated by increased active ERK 1/2 in knock-in cells. Scale bars represent 100 µm (B) and 50 µm (F), Error bars represent mean +/- S.D. (B, D, E, G, H, K) and mean +/- S.E.M (I, J). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

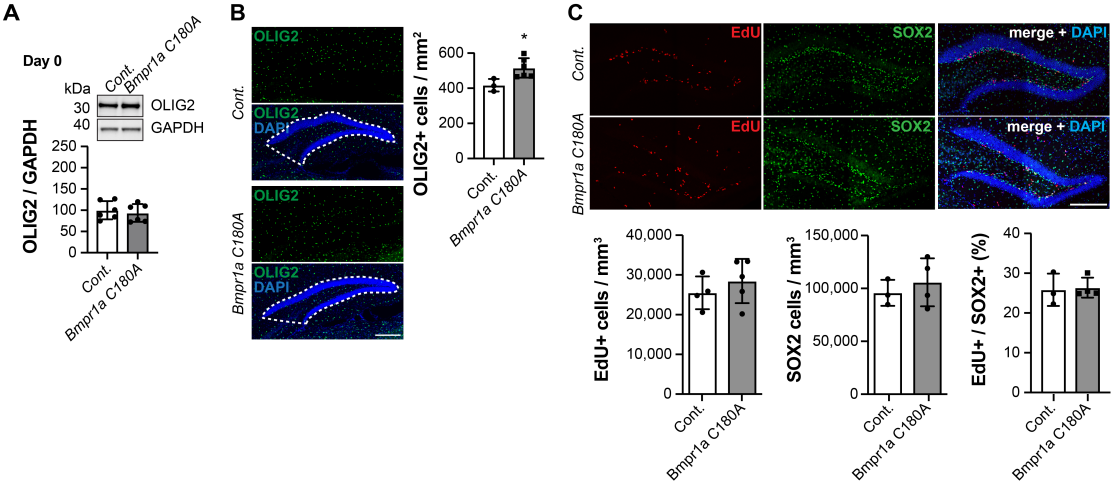


Fig. S4. BMPR1a C180A does not affect proliferation of SOX2+ cells in the adult brain. (A) OLIG2 expression is not changed between control (white) and *Bmpr1a C180A* (grey) knock-in NSCs under proliferating conditions *in vitro.* (B) The density of OLIG2+ cells (green) in the hippocampus is increased. Nuclei were counterstained using DAPI. (C) The ratio of EdU (red) / SOX2 (green) co-labeled cells is comparable between control (white) and *Bmpr1a C180A* (grey) mice. Scale bars represent 200 µm (B, C). Error bars represent mean +/- S.D. *$p < 0.05$

Supplementary Table legend

Table S1. Mass spectrometric-based identification of proteins after acyl-biotin exchange and streptavidin-based enrichment.

Sheet 1: Raw data exported from Progenesis QI for proteomics. Listed are the p-values (column E), q-values (column F) and the maximum fold change (column G) of potentially S-acylated candidate proteins. Shown are also the raw abundances for hydroxylamine treated (column R-T) and control samples (column U-W). As well as the normalized abundances for hydroxylamine treated (L-N) and control samples (O-Q) after the normalization to housekeeping proteins. Sheet 2: Identified candidate proteins. The normalized abundances of hydroxylamine-treated (column F-H) and control conditions (column I-K) were log2 transformed and the log2 change was calculated (column L). Identified proteins were considered as potentially S-acylated candidate proteins if the p-value was <0.05 and the log2 change was higher then two (column M-N). Note: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014355.

Supplementary Materials and Methods

Cloning

For the generation of overexpression constructs, cDNA sequences of interest were exported from Ensembl genome browser (ensembl.org) and sequence specific primers were obtained from Microsynth. NSCs were cultured as described before and total RNA extraction was performed using the PureLink RNA Mini Kit. The extraction was performed by following the manufacturer's instructions. Obtained mRNAs were stored at -80°C and 5 µg of total RNA was used for the generation of cDNAs. cDNA generation was performed by using the SuperScript III First-Strand Synthesis System. cDNA was generated by following the manufacturer's instructions (random hexamers were used). In order to amplify the construct of interest, elongation time and annealing temperature were adjusted for the individual PCR constructs and primers and 1µl of cDNA was used as template. Overexpression constructs were generated by introducing the sequence of interest into the CAG-GFP vector. To introduce point mutations, two separate PCR reactions were performed, one upstream of the target sequence and the second one downstream of it. For these PCR reactions primers were used that contained the desired sequence as an overhang on the primer. The two separate PCR reactions were then introduced into the target vector by Gibson Assembly. For all PCR reactions Phusion polymerase was used. All PCR products and digested plasmids, used for downstream processing, were gel purified using the QIAquick gel extraction kit. Individual PCR pieces and plasmids were assembled using Gibson Assembly, by following the manufacturer's instructions. For all transformations DH5 competent E. coli were used. Transformations and minipreps were performed, according the manufacturer's instructions.

Gene editing

CRISPR/Cas9 gene editing was performed as described before(1). Nucleotide sequences for the gene of interests were downloaded from Ensembl genome browser. Genomic DNA and cDNA sequences were exported as FASTA files and processed with ApE1. gRNAs targeting either the 5'-end of the coding

sequence of the gene of interest or its 3'-end, for knockout or tagging, respectively, were designed using the online tool at crispr.mit.edu/. The corresponding sense and antisense oligonucleotides were synthesized at Microsynth and hybridized to a duplex in a reaction mixture composed of 1 µl of each of the 100 µM oligonucleotides, 1 µl 10x T4 DNA ligase buffer with 10 mM ATP, 1 µl T4 PNK, and 6 µl distilled H_2O, on a thermocycler using the following settings: 37 °C for 30 min, 95 °C for 5 min, followed by a stepwise decrease in temperature to 25 °C (5°C / 1 min). 2 µl of the annealed oligonucleotides were then used in a ligation reaction with 10 ng of BbsI-digested and gel-purified pSPCas9n(BB)-2A-Puro plasmid, 2 µl T4 DNA ligase buffer with 10 mM ATP, and 1 µl T4 DNA ligase, supplemented to 20 µl with distilled H_2O, at room temperature for 30 min. 2 µl of the ligation reaction was used for transformation of E. coli. The successful integration of the gRNA sequence into the plasmid was confirmed by sequencing. For the HDR plasmid we used pfa6 as a backbone. The inserted sequence contained the following information in the indicated order: 1kb upstream homology sequence for the gene of interest, linker sequence, sequence encoding for the fluorophore of interest or HA-tags, T2A sequence, antibiotic resistance gene and downstream 1kb homology sequence. Silent mutations were introduced to mutate the PAM sites. Single PCR fragments were assembled using Gibson Assembly. Plasmids of interest were introduced into the target cells by electroporation (NSCs) or lipofection (ESCs), followed by an antibiotic selection over a time period of at least two weeks (100 µg/ml G418 for NSCs and 300 µg/ml for ESCs). For CRISPR/Cas9 mediated knockout experiments, two different gRNAs, targeting the same gene were used. Control cells were always electroporated or lipofected with non-targeting gRNAs. gRNAs used in this study:

gRNA Bmpr1a F (tagging): ATTGTCAAATCTTTACATCC
gRNA Bmpr1a R (tagging): GGATGTAAAGATTTGACAAT
gRNA Nup210 F (tagging): CCTCTCACTAGGCAATGTGC
gRNA Nup210 R (tagging): GCACATTGCCTAGTGAGAGG
gRNA Bmpr1a KO F:ATACACTTACATCAGATTAC
gRNA Bmpr1a KO R: GTAATCTGATGTAAGTGTAT

gRNA Bmpr1a KO F: TAAGGTTGTAGTGATATGGC
gRNA Bmpr1a KO R: GCCATATCACTACAACCTTA
gRNA Bmpr1a F C180A (mouse): ATTGTAAGAGTATCTCAAGC
gRNA Bmpr1a R C180A (mouse): GCTTGAGATACTCTTACAAT
gRNA Bmpr1a F C173/175A (mouse): TTACTTATAGCAAAAGCAGC
gRNA Bmpr1a R C173/175A (mouse): GCTGCTTTTGCTATAAGTAA
gRNA nT F: GCACTACCAGAGCTAACTCA
gRNA nT R: TGAGTTAGCTCTGGTAGTGC

Electroporation and Transfection

NSCs were cultured as described above. Prior to the electroporation poly-L-ornithine, laminin coated dishes were prepared and equilibrated with fresh media in the incubator. Immediately before the electroporation the cell pellets (1 x 10^7 cells) were collected by centrifugation (150g / 1min) and dissolved in 100µl (room temperature) electroporation solution + plasmid DNA of interest. The electroporation was performed using the AMAXA electroporation system (Lonza) with the electroporation program A-0033 (mouse NSC). After the electroporation the cells were immediately transferred to the pre-equilibrated plates. For lipofections cells were seeded on poly-L-ornithine, laminin coated plates or coverslips. Fresh media was added on the day of the experiment. Two different mixes were prepared: DNA was added to 50 µl Opti-Mem and second Lipofectamin 2000 was added to 50 µl Opti-Mem. Both were incubated for 5 min at room temperature, before mixing them together. After another 20 min of incubation, the solution was added drop wise to the cells. Different DNA amounts were used for different plate sizes (0.25 µg for 12 wells, 1 µg DNA for 6 wells).

SDS PAGE & Western Blot

Protein isolation from adherent NSCs or ESCs was performed using RIPA as describe before (2). Protein concentrations were determined using the Biorad DC assay by following the manufacturer's instructions. MOPS or MES running buffer was used. The gels were run at a constant voltage of 150 V. Protein transfer was performed for high-molecular weight proteins using PVDF membranes at 40 V for 2h using the Nupage transfer system. For low-

molecular weight proteins the Trans-Blot Turbo system and Trans-Blot Turbo Mini PVDF transfer packs were used. Membrane blocking was performed with 3 % BSA in TBST (TBS + 0.05% Tween) for 1h. Blots were incubated with primary antibodies overnight in 3 % BSA in TBST. On the next day blots were washed 3 x 10 min in TBST before incubation with secondary antibodies for 1h in 3 % BSA. Blots were then washed three more times with TBST. Chemiluminescence was developed using the supersignal west pico or femto kit. Fluorescence imaging was performed on a Biorad gel imaging system or on a LICOR infrared scanner (17-ODYA labeling experiments). Quantification was performed using Fiji imaging analysis software and Image Studio Lite. Primary antibodies used were: GFP (1:3000, Rockland), Streptavidin-647 (1:5000, Thermo Fisher Scientific), HA (1:3000, Abcam), ACTB (1:3000, Sigma Aldrich), BMPR1A (1:500, R&D), GAPDH (1:3000, HyTest), Beta Dystroglycan (1:100, Santa Cruz), P-p44/p42 (1:1000, Cell Signaling), P-SMAD1/5 (1:1000, Thermo Fisher Scientific), OLIG2 (1:1000, Millipore), NG2 (1:1000, Millipore), TUBA4A (1:2000, Abcam).

Luciferase assays

For luciferase assays, the Dual-Luciferase Reporter Assay kit was used, by following the manufacturer's instructions. All experiments were performed at least in triplicates. NSCs were electroporated with the constructs of interest as described above and seeded on coated 6 well plates. To measure the activity of BMP signaling, we used a BRE-luc reporter construct, together with the control reporter plasmid pTK-RL, to normalize for differences in electroporation efficiency. Plates were measured on a NOVOstar microplate fluorometer (BMG Labtech).

Cell proliferation analysis

Seeded cells were incubated for 1h with a final concentration of 10 µM EdU (5-ethynyl-2'-deoxyuridine), before fixation with 4% (w/v) PFA. For the click reaction Click-iT EdU Alexa Fluor 647 imaging kits were used. Click reactions were performed following the manufacturer's instructions. All experiments were performed at least in triplicates. DAPI (4 ,6-Diamidin-2-phenylindol) was used as a counterstain. Pictures were taken on a confocal microscope (Zeiss

LSM 800) or a fluorescence microscope (Zeiss AxioObserver Z1) and analyzed by using Fiji.

IP-ABE assay

IP-ABE assays were performed as previously described (3). NSCs were electroporated with constructs of interest. After 24h cells were lysed with 300 µl lysis buffer (LB) + NEM: (50 mM NEM), 50 mM Tris pH 7.4, 150 mM NaCl, 10 % glycerol, and 1 % IGEPAL, 1 mM PMSF and proteinase inhibitors by going through a 26 gauge syringe. Lysates were transferred to precooled tubes and incubated for 30 min on ice. After a 20 min centrifugation step at 16000 g the supernatant was collected and incubated with 50 µl of bead slurry (magnetic-HA beads or non magnetic-HA affinity matrix) for 3h at 4 °C with LB + NEM. Beads were then washed 1x with stringent buffer (10 mM NEM, 0.1% SDS, 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 % IGEPAL, 1 mM PMSF and proteinase inhibitors and three more times with LB (without NEM), before they were equally divided to two 1.5 ml tubes and treated with + HAM (50 mM Tris pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 M Hydroxylamine and – HAM buffer for 1h at room temperature (RT). Beads were then washed once with LB, before incubation with 5 µM Biotin BMCC-buffer. After 1 h incubation at room temperature, beads were washed three more times with LB. To elute the proteins, samples were incubated for 10 min at 85 °C with 2x SDS buffer. Samples were then supplemented to a final concentration of 50 mM with DTT. SDS-Page and Western Blot were performed as described above. The biotin was then detected using streptavidin 647 antibodies and the protein of interest with anti-HA (Abcam) or anti-GFP (Rockland) antibodies. All experiments were performed at least in triplicates.

17-ODYA labeling

NSCs were electroporated with the construct of interest as described above. On the day of the experiment NSCs were washed 1x with 37 °C DPBS and incubated for 3h with palmitate (C16) or 17-ODYA (Palmitate ortholog 17-octadecynoic acid carrying an alkyne group). After treatment cells were washed twice with ice-cold DPBS and lysed with 500 µl lysis buffer (without proteinase inhibitor and without PMSF). Lysates were then used for

immunoprecipitation (60µl magnetic anit-HA beads), followed by three sequential washings with lysis buffer and two additional washing steps using PBS. Beads were then suspended in 44 µl PBS. The Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) links the IR-800 dye to the 17-ODYA lipid: Following reagents were added in the indicated order: 1 µl CuSO4 50 mM stock solution, 1 µl TCEP (Tris(2-carboxyethyl)phosphin) 50 mM stock solution, 3 µl TBTA (Tris(benzyltriazolylmethyl)amine) 1x working solution and 1 µl near-infrared dye IRDye 800 CW (IR-800) stock solution. The IR-800 dye carries an azide group. The reaction was performed in the dark and stopped by the addition of 4x loading buffer after one hour. Samples were immediately used for SDS PAGE and Western blot analysis. All experiments were performed at least in triplicates.

Acyl-PEG-exchange

NSCs were electroporated with constructs of interest as described above. After 24h cells were lysed in 100 µl of LB + NEM pH 7.2 by going through a 26 gauge syringe and supplemented to a final concentration of 1.7 % with Triton-X-100 and to 1 % with Chaps before incubation on ice for 30 min. Centrifugation with 17000g for 10 min was performed and supernatant was collected and precipitated once by C/M precipitation (chloroform / methanol). Pellets were dried by using a centrivap (Labconco) and suspended in SDS-LB + TCEP (50 mM TrisHCL pH 7.4, 10 mM TCEP, 5 mM EDTA, 4 % SDS. Pellets were then solubilized by sonication for 5 min at 37 °C. TCEP was removed using C/M precipitation and pellets were suspended in 37.5 µl SDS-LB + NEM and diluted with 112.5 µl dilution buffer (50 mM TrisHCL pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2 % Triton-X-100, 2 mM PMSF and proteinase inhibitors, before overnight incubation at 4°C. NEM was removed by three sequential C/M precipitations. After the last precipitation pellets were suspended in 50 µl LB-SDS and divided into two tubes (25 µl each). 125 µl + HAM and – HAM buffer was added and incubation was performed for 1h at room temperature One more C/M precipitation was performed and pellets were suspended in 30 µl SDS-LB. 120 µl mPEG solution (60 mM Tris, 5 mM EDTA, 0.2 %-Trition-X-100, 20 mM mPEG (5 kDA or 10 kDA) was added to the samples. Incubation was performed for 2h at room temperature. An

additional C/M precipitation was performed before suspending samples in 30 µl LB. All experiments were performed at least in triplicates. Before performing SDS-PAGE and Western Blot analysis, samples were stored at -20°C.

Acyl-Biotin-Exchange assay for mass spectrometric analysis

The protocol was performed as previously described with some minor modifications (Wan, et al., 2007): NSCs were cultured on poly-L-ornithine and laminin coated 10 cm cell culture plates. Prior to protein extraction cells were washed twice using 4 °C cold PBS. Lysis was performed using 250 µl LB. Lysates were supplemented with Triton X-100 to 1.7 % and with Chaps to 1 % and incubated for 1h end-over-end at 4 °C. Samples were centrifuged at 250g for 5 min at 4 °C. Protein concentration was measured using Biorad DC assay according to the manufacturer's instructions. Samples were adjusted to the same volume and concentration (5 ml, 2.2 mg/ml) by the addition of LB. Samples were then precipitated once using C/M precipitation and pellets were resolved in 2.5 ml LB pH 7.4 at 37 °C for 30 min by gentle agitation. Samples were diluted with dilution buffer pH 7.2 at 4 °C and 1 mM PMSF to 10 ml and incubated overnight with gentle agitation at 4 °C, followed by three sequential C/M precipitations. Samples were then divided into a + HAM and a – HAM sample and incubated for 1h with end-over-end rotation at room temperature. After an additional CM precipitation samples were incubated for 1h with low-HPDP binding buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.2 mM HPDP–biotin, 0.2 % Triton X-100, 1 mM PMSF, PI. Samples were cleaned two more times with C/M precipitations and frozen down at -20 °C. Next day the samples were thawed and precipitated once more with C/M and suspended in LB pH 7.2. Samples were diluted 1:20 to a final SDS concentration of 0.1 % with dilution buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.2 % Triton-X-100, 1xPI, 1 mM PMSF and centrifuged 16000g for 5 min. A Biorad DC assay was performed and protein concentrations were equalized before incubation with streptavidin agarose for 90 min at room temperature After incubation beads were washed 5 times with LB and 50 mM for 15 min at 37 °C with occasional gentle mixing, followed by TCA precipitation (10 %) for 1h at ice. Samples were centrifuged for 20 min at 4 °C

and washed twice with -20 °C acetone. Pellets were dissolved in LB pH 8.2 (4 % (w/v) SDS, 100 mM Tris, 100 mM DTT) and stored at -80 °C.

Methanol chloroform precipitation

For larger scale precipitations up to 5ml of sample was substituted with 4 volumes of methanol, 1.5 volumes of chloroform and 3 volumes water, vortexed and centrifuged for 45 min at 4000 g. The supernatant was removed and 4 volumes of methanol were added to the protein pellet. Followed by 15 min centrifugation at 4000 g. The supernatant was again removed, the pellets were air dried and suspended in the buffer of interest by brief sonication in a 37 °C water bath. For small scale precipitations up to 150 µl of sample was substituted with 4 volumes of methanol, 1.5 volumes of chloroform and 3 volumes water, vortexed and centrifuged for 5 min at 17000 g. The supernatant was removed and 4 volumes of methanol were added to the protein pellet. Followed by 2 min centrifugation at 17000g. The supernatant was again removed, the pellets were air dried in a vacuum dry evaporator (Labconco), connected to a -80 °C cold trap (Labconco), and suspended in the buffer of interest by brief sonication in a 37 °C water bath.

Analyzing receptor dimerization by co-immunoprecipitation

HA-tagged BMPR1a was overexpressed together with FLAG-tagged BMPR1a or BMPR2 in NSCs by electroporation as previously described. 15h later, the cells were lysed with PBS + 1 % Triton X100 + 150 mM NaCl + proteinase inhibitors, protein concentration of the post-nuclear fractions was measured as described above, and 300 µg of protein were subjected to immunoprecipitation with pre-blocked (3 % BSA in lysis buffer, 1h) anti-HA magnetic agarose (Thermo Fisher Scientific) for 15h at 4 °C. The beads were washed three times with lysis buffer with 250 mM NaCl, before elution of the proteins by heating at 95 °C for 5 min in 1x SDS lysis buffer and subsequent reduction by addition of DTT to 100 mM for 15 min. The whole samples were used for SDS-PAGE and Western blotting as described above.

Cell-surface expression

Experiments were performed as described before (4). For measuring the cell

surface expression of proteins, E17.5 NSCs and E17.5 NSCs *Bmpr1a c180a* were seeded on poly-L-ornithine, laminin coated 6 well plates (Nunc). Cells were removed from the incubator, incubated on ice, and washed twice with CM-PBS (4 °C) on ice. Cells were then incubated for 30 min with 2.5 ml biotin buffer (0.5 mg/ml, sulfo-NHS-LC-biotin) on ice and washed with CM-PBS. Then cells were washed twice for 15 min with quenching buffer (50 mM NH4Cl, 1 % BSA in CM-PBS). After that two additional washing steps with 5 ml CM-PBS were performed. Cells were then lysed using 450 µl lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 % glycerol), and 1 % IGEPAL, followed by a 30 min end-over-end incubation at 4 °C. After incubation a 10 min centrifugation step at 17000g at 4 °C was performed. 50 µl of the supernatant was saved as an input control and the rest of the sample was used for immunoprecipitation. Samples were incubated for 1.5h with magnetic streptavidin beads and washed three times with lysis buffer. Proteins were then eluted by the addition of lysis buffer with LDS sample buffer and 10x reducing agent and heated to 80 °C for 10 min before performing SDS PAGE and Western blotting. To characterize the differences in the surface expression of BMPR1a and BMPR1a-C180A and to compensate for differences in the biotinylation or immunoprecipitation efficiency both samples were normalized to the internal control ß-dystroglycan (ß-DG).

Measuring endocytosis

For measuring the internalization rate of cell surface proteins, NSCs were seeded onto 6 wells plates (2 plates for each condition), removed from the incubator and directly placed on ice, washed twice with CM-PBS (4°C) and incubated for 30 min with 2.5 ml biotin buffer on ice. Unbound biotin was then removed by an additional wash with CM-PBS. New medium was added before plates were shifted back for indicated times to 37 °C. After the incubation, plates were transferred back to ice and immediately washed once with ice cold CM-PBS. Followed by two 15 min incubation steps with 2.5 ml reducing buffer (50 mM reduced glutathione, 75 mM NaCl, 75mM NaOH) on ice. After the reduction step, cells were washed twice with 2.5 ml quenching buffer (50 mM Iodoacetamide, 1 % BSA in CM-PBS) and washed two additional times with CM-PBS. Cells were then lysed on ice with 450 µl lysis

buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, and 1% IGEPAL). As we used two plates for each condition, the two plates were pooled after lysis. Lysis, immunoprecipitation, SDS PAGE and Western blot analysis were performed in a similar way as for the cell-surface expression analysis. To compensate between reaction efficiencies all samples were normalized to the internal control ß-DG, a cell surface protein that undergoes endocytosis.

Fluorescence-recovery after photobleaching (FRAP)

Zeiss Confocal microscope LSM800 was used to perform FRAP. Bleaching was performed with 488nm laser at 15% laser power with 80 iterations. First, 5 images were taken pre-bleach. Then the bleaching was performed for approximately 2.4 seconds followed by 95 seconds of image acquisition. The recovery in the bleached area was measured using ImageJ. All the intensities were then normalized to the average of the first 5 frames. The data was then fitted to the equation $I = I0 + (R – I0) \times (1 – e(-kt))$ where I is the intensity, I0 is the intensity at time = 0 seconds, R is the total recovery and t is time in seconds. The total recovery R is then compared between the different conditions.

EdU injection

For analysis of developmental oligodendrogenesis at postnatal day 7 (P7), pregnant *Bmpr1a C180A* and wild type female mice at E17.5 were injected once intra-peritoneally with 50 µg/kg body weight BrdU in 0.9 % (w/v) NaCl solution. For studies of proliferation in adult mice, two month old male and female *Bmpr1a C180A* and wild type mice were injected once per day for three consecutive days intra-peritoneally with 100 µg/kg body weight EdU in 0.9 % (w/v) NaCl solution. The mice were sacrificed and their brains analyzed 24 hours after the last injection.

Immunohistochemistry

E15.5 and E17.5 embryos were dissected in cold dissection solution composed of 15.6 g DMEM/F-12 (1:1) powder, 15 mM $NaHCO_3$, 10 ml penicillin-streptomycin-amphotericin-B, and 16 mM D-glucose, supplemented

to 1 l with distilled H_2O. The embryonic brains were fixed for 4 or 5 hours (for E15.5 and E17.5, respectively) at 4 °C with cold 4 % (w/v) PFA in PBS, equilibrated overnight at 4 °C in cold 15 % (w/v) sucrose in PBS and then again overnight at 4 °C in cold 30 % (w/v) sucrose in PBS, before being frozen in O.C.T. compound (Tissue-Tek®) using liquid nitrogen. The frozen brains were sectioned into 20 µm coronal sections on a cryostat and stored at -20 °C until staining for immunofluorescence microscopy. The slides with frozen embryonic brain sections were dried at room temperature, after which the sections were encircled with ImmEdge tissue pen. After letting the marker dry for 20 min, the slides were washed three times, for 10 min each, with PBS. Blocking was achieved by incubating the sections for 1.5h at room temperature covered with droplets of PBS with 10 % (v/v) donkey serum and 0.5 % (v/v) TritonX-100 (hereinafter referred to as PBS++). Subsequently, the sections were incubated twice overnight (for about 44 hours) covered with droplets of the primary antibodies in PBS++. The sections were then washed three times, for 10 min each, with PBS, blocked again for 30 min at room temperature with PBS++, and stained for 1.5h at room temperature with droplets of the secondary antibodies together with DAPI (diluted 1:10,000) in PBS++. Finally, the sections were washed again three times for 10 min with PBS and then mounted with ImmuMount. P7 pups and two month old male and female mice were anaesthetized by intra-peritoneal injection of 225 mg/kg body weight pentobarbital (Esconarkon), followed by trans-cardial perfusion with 0.9 % (w/v) NaCl in H_2O for 5 min and 0.4 % (w/v) PFA in PBS for 2 min after the tail starts to move. The brains were dissected and post-fixed for 16 hours at 4 °C in 4 % (w/v) PFA in PBS, followed by dehydration for 48 hours at 4 °C in 30 % (w/v) sucrose in PBS. Subsequently, the brains were frozen and sectioned into 40 µm coronal sections on a sliding microtome, the sections were stored at 4°C in CPS (0.05 M phosphate buffer + 25 % (v/v) glycerol + 25 % (v/v) ethylene glycol).

Free floating brain sections were washed for 3 x 10 min with TBS, before blocking with TBS + 0.25% Triton-X-100 + 3% donkey serum for 30 min. Staining for MBP was performed using 0.5 % TritonX-100 in the blocking buffer. Sections were then incubated for 48h with primary antibodies at 4 °C.

After two sequential washing steps with TBS, sections were blocked once more for 15 min at room temperature (RT), followed by incubation with secondary antibodies (1:500, Jackson Immuno Research). DAPI was used for counterstaining. Primary antibodies used were SOX2 (1:200, Santa Cruz), SOX2 (1:500, Millipore), NESTIN (1:500, BD), TBR1 (1:200, Abcam), BMPR1A (1:100, Thermo Fisher Scientific), OLIG2 (1:500, Millipore), NEUN (1:500, Millipore), NG2 (1:350, Millipore), APC (1:500, Millipore), MBP (1:500, Serotec), BrdU (1:200, Abcam). Images were acquired on a Zeiss LSM800 confocal microscope or a Zeiss Axio ScanZ1 slide scanner.
Sections were mounted using Immu-Mount. For EdU-injected mice, click reaction was performed using the Click-iT EdU Alexa Fluor 647 imaging kit and following the manufacturer's protocol, except for the substitution of the blocking buffer by TBS + 0.25% Triton-X-100 + 3% donkey serum. Images were acquired on a Zeiss LSM800 confocal microscope or a Zeiss Axio ScanZ1 slide scanner.

Immunocytochemistry

Cells were seeded on glass coverslips and fixed with 4 % (w/v) PFA. After fixation, cells were washed 1 x with PBS and 1 x with TBS. Blocking was performed using TBS (supplemented with 3 % donkey serum and 0.25 % Triton-X-100) and primary antibodies were incubated overnight at 4 °C. The next day, cells were washed twice with TBS, blocked for another 15 min and incubated for 1.5h with secondary antibodies. DAPI was used as a counterstain (1:5000). Coverslips were mounted using Immu-Mount. For analysis of plasma membrane proteins, the normal TBS++ was substituted by TBS with 0.5 % (w/v) BSA and 0.1 % (v/v) TritonX-100. Primary antibodies used were GFP (1:500, Rockland,), HA (1:600, Cell Signaling), OCT4 (1:500, Santa Cruz), NANOG (1:500, Abcam), BMPR1A (1:100, Thermo Fisher Scientific), NESTIN (1:500, BD).

Droplet Digital PCR

A total RNA extraction was performed using the PureLink RNA Mini Kit in combination with an on-column DNAse digestion (PureLink DNase Set), following the manufacturer's instructions. Obtained mRNAs were stored at

-80 °C. For proliferating E17.5 NSCs 2 µg RNA was transcribed into cDNA (For differentiated cells 200 ng), by using the SuperScript III First-Strand Synthesis System. Random hexamers were used for the cDNA generation. cDNA synthesis was performed by following the manufacturer's instructions. Before droplet generation a reaction mix (25 µl / reaction) containing 260 nM gene specific primers, cDNA corresponding to 25 ng RNA and QX200 ddPCR EvaGreen was mixed together. 20 µl of the mix was then transferred for droplet generation into a DG8 cartridge, 70 µl oil for droplet generation was added and droplet generation was started on a QX200 Droplet Generator (Biorad). After completion, the reactions were transferred to a PCR plate (Biorad), which was heat-sealed using a PX-1 PCR Plate sealer (Biorad). PCR reactions were performed in with a SensoQuest labcycler gradient using following settings: 95 °C for 5 min, 50 cycles of 95 °C for 30 sec followed by 60 °C for 1 min, 4 °C for 5 min, 90 °C for 5 min, hold at 4 °C. After the PCR run was completed, droplets were analyzed immediately on a QX200 droplet reader (Biorad). Data analysis was performed using QuantaSoft software (Biorad). Following gene specific primers were used:
ID1 F: CCAGTGGGTAGAGGGTTTGA, ID1 R:
AGAAATCCGAGAAGCACGAA,
ID2 F: ACCCGATGAGTCTGCTCTAC, ID2 R: CTGGTTCTGTCCAGGTCTCT,
ID3 F: AGGTGTCTCTTTTCCTCCCT, ID3 R: ATGTCGTCCAAGAGGCTAAG,
ID4 F: GAGACTCACCCTGCTTTGCT, ID4 R: AGAATGCTGTCACCCTGCTT

Image analysis

Image analysis was performed using ImageJ, ZEN 2 (Carl Zeiss), or Imaris (Bitplane). All quantifications were performed in a blind manner. For the quantification of the mNeonGreen intensity in the TBR1-positive layer in electroporated E15.5 embryonic brains, a mask of the TBR1-positive layer was generated manually, before analysis of the raw mNeonGreen intensity in the TBR1-positive mask and in the whole ROI. Measurement of the cortical gray matter area in P7 brains was performed in 7 coronal sections along the rostral – caudal axis, ranging from the beginning of the lateral ventricle (primary motor area) to the end of the DG (primary visual area). For the quantification of cell numbers in P7 brains, areas containing the neocortex

and the corpus callosum were defined and extracted a priori using Fiji. All images were then exported to Imaris software (Bitplane) for image analysis. Number of OLIG2 and BrdU positive cells were counted semi-automatically using Imaris' algorithm to create defined spots with the same intensity and defined 7um diameter, followed by manual correction of the obtained counts. Upon generation of spots for both markers, co-localization was determined using the *Spots Colocalize* Matlab plugin with a defined distance between centers of spots of 6um.

For the analysis of cell numbers in two months old DG, the cells were counted in either the SGZ only or in the entire DG including the hilus in 5 to 7 coronal sections spanning the whole DG. The cell counts were then normalized by the DG volume, or the DG area for slide scanner images. Cell numbers in two months old cortex were counted in the dorso-medial cortical gray matter or in the corpus callosum, in two (OLIG2 and NEUN counting) or three (NG2 and APC counting) coronal sections located at the level of the primary motor area and the primary somatosensory area. Cell counts were normalized by the analyzed area.

Generation of knock-in mice

The (C180A) modification of the Bmpr1a locus using Cas9/CRISPR was carried out directly in mouse one-cell embryos by electroporation. Electroporation with a mixture of 16uM cr:trcrRNA hybrid targeting bmpr1a, 16uM Cas9 protein and 200nt ssDNA oligonucleotide carrying the desired mutations (all reagents from IDT) was carried out using the ECM830 electroporator (BTX Harvard Apparatus). Two square 3 ms pulses of 30V with 100 ms interval were applied as previously described (5). Surviving embryos were transferred immediately into the oviducts of 8–16-wk-old pseudopregnant Crl:CD1(ICR) females that had been mated with sterile genetically vasectomized males (6) the day before embryo transfer (0.5 dpc).

References (SI)

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