SUPPLEMENTAL MATERIALS AND METHODS

Plasmids and Constructs

For pre-miRNA cloning into the pcDNA3.1 vector, approximately 150 base pairs were included in the 5' and 3' region of the stem loop sequence (obtained from www.mirbase.org). Mutations were introduced into the miRNAs' seed sequence and the predicted binding sites within the Ncad 3'UTR using the QuickChange II Site-Directed Mutagenesis kit (Stratagene). All primer sequences are annotated in Table S2. The pNeuroD1-mCherry reporter and the pNeuroD1-NcadHA plasmids were generated by cloning the mCherry, or NcadHA, cDNAs downstream of the NeuroD1 promoter (Beta2) in the pBS-Beta2 plasmid. The pBS-Beta2 plasmid was kindly provided by Ming-Jer Tsai.

in utero electroporation

Briefly, embryonic day E13.5 pregnant mice were anaesthetised with isoflurane, and the uterine horns were exposed. For overexpression studies, miRNA expression vectors (1.5 μg/μl) were injected with either pCAG-eGFP or pCAG-mCherry (0.5 μg/μl) into the lateral ventricle of each embryo. For rescue experiments, miRNA expression vectors were injected with either pCAG-eGFP or pCAG-mCherry (0.125 μg/μl) and pCAG-NcadHA (0.125 μg/μl). For knockdown studies, miArrest synthetic miRNA inhibitors (LNA anti-miRs) were purchased from GeneCopoeia. 320 pmol of LNA anti-miRs were injected with 0.5 μg/μl of pCAG-mCherry. For Ncad HA-tagged overexpression, pCAG-NcadHA (1.5 μg/μl) was injected with pCAG-mCherry (0.5 μg/μl). For electroporation,

ten pulses of 40 V for a pulse length of 50 ms at 950-ms intervals were applied (Electro Square Pavator, BTX; Harvard Apparatus). The uterine horns were returned to the abdominal cavity, and the embryos were allowed to develop for 48-96 hours. For analysis, mice were sacrificed by cervical dislocation, and the embryos were collected. The brains were isolated, fixed in 4% paraformaldehyde (PFA) overnight at 4°C, and then incubated in a 15% sucrose and 30% sucrose solution for cryoprotection. The brains were finally embedded in Cryomatrix (Thermo Scientific) and snap frozen. For Ncad immunostaining, 10-μm-thick brain slices were collected. For cell counting and all other immunostaining experiments, 20-μm-thick brain slices were collected.

Immunohistochemistry

For immunohistochemistry, brain sections were fixed in 4% PFA at room temperature (RT) for 10 minutes. After two washes with PBS, an antigen retrieval treatment was performed by embedding the sections in 10 mM sodium citrate with 0.5% Tween-20, pH 6, at 80°C for 20 minutes. The sections were then blocked in PBS with 0.2% Tx-100 containing 2% sheep serum. Primary antibody incubation (rabbit Anti-Pax6, rabbit Anti-Tbr1, rabbit Anti-Tbr2 from Abcam; 1:500, mouse Anti-Ncad from BD Transduction Laboratories; 1:1000) was performed in blocking solution overnight at 4°C. The sections were washed three times in PBS 0.2% Tx-100 and incubated in blocking solution containing Alexa-488- or Alexa-594-conjugated secondary antibody (Invitrogen; 1:500) for 4 hours at RT. After three washes in PBS, the sections were embedded in DABCO-DAPI (1:1000) medium.

The sections were viewed using a laser-scanning confocal microscope (LSM780), and pictures were taken with the ZEN 2010 software (Zeiss). The images were analysed, and the cells were counted using ImageJ software. Pictures from at least three different electroporated brains were analysed for each treatment.

Fluorescence-activated Cell Sorting (FACS)

Electroporated brains were dissected, and the tissue was disrupted by pipetting in PBS with 0.5 mM EDTA. For cell surface Ncad staining, living cells were incubated in a mouse anti-Ncad antibody (BD Transduction Laboratories) at a concentration of 1:500 in PBS with 10% FCS. After 1 hour of incubation at 4°C in the dark, the cells were centrifuged at 300×*g* and washed with PBS. The cells were then fixed in 1% PFA with 0.1% sodium citrate at RT for 10 minutes and then made permeable with 0.1% Tx-100 in PBS for 10 minutes. The cells were then washed and incubated in PBS with 10% FCS, and the indicated primary antibodies (rabbit Anti-Pax6, rabbit Anti-Tbr1, rabbit Anti-Tbr2 from Abcam; 1:500) for 1 hour at 4°C. After washing, the cells were incubated with secondary antibody (anti-mouse-Alexa488, anti-rabbit-Cy5, Invitrogen; 1:500) for 30 minutes at 4°C. FACS analysis was performed with a LSR II Cytometer (BD) immediately after treatment.