Antibodies to use:

KCC2 – 1:500, Rabbit polyclonal, 07-432, Millipore
NKCC1 – 1:500, Rabbit polyclonal, ab59791, Abcam
Parv – 1:2000, Mouse monoclonal, SAB4200545, Sigma
Donkey α-mouse AF488 – 1:200, 715-545-151, Jackson ImmunoResearch
Donkey α-rabbit Cy3 – 1:200, 711-165-152, Jackson ImmunoResearch
Donkey α-mouse Cy3 – 1:200, 715-165-150, Jackson ImmunoResearch
Donkey α-mouse Cy5 – 1:200, 715-165-151, Jackson ImmunoResearch

Perfusion: Perfusion fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4, and 15% saturated picric acid. This fixative will be good for several weeks at 4°C.

Post fix brains for 1-2 days in fixative at 4°C, then transfer to 0.1 M PB + 0.1% sodium azide

Slicing: Tissue sections from fixed brains are generated by a vibratome, ideally at 40 μm thickness. Collect horizontal section that contain the thalamus in cryoprotectant (see below) and store at -20°C. Section will remain good for up to several years.

Cryoprotectant

500mL 0.1 M PB
300mL ethylene glycol
200mL glycerol

PBS

0.1 M PB
0.9% NaCl

Incubation Buffer (store at 4°C)

PBS
0.1% Sodium azide
1% Normal goat serum
for this protocol
(0.1% Triton when added)

Tris Buffer

0.05 M Tris HCl

All incubations are interspersed with triple washes done in PBS throughout the entire procedure, by transferring sections between wells.

All blocking and antibody solutions were prepared in "incubation buffer"
Blocking and antibody solutions are recovered after use and stored (at 4°C) for future use.

About 5 ml of solution needed per well in a 6 well plate.

Remove sections from cryoprotectant and wash in PBS. Cryoprotectant is highly viscous, so it may take several rinses to completely remove it

2.1 Sodium borohydride treatment. Add sodium borohydride ($NaBH_4$) to PBS (1 mg/ml). Transfer sections into 0.1% sodium borohydride for 15 minutes. Bubbles will appear and sections tend to float.

2.2 Wash: 2x wash in PBS, 5 minutes, make sure to remove bubbles.

2.3 Blocking solution: Add to incubation buffer: 2% normal goat serum (20 µl/ml) and Fab' fragment of goat anti-mouse IgG (1:500 dilution: 2 ul/ml). Incubate for 4 hours at RT or overnight at 4°C.

2.4 Wash: 3x wash in PBS, 5 minutes.

3.1 Make up appropriate primary:

KCC2+Parv: Dilute rabbit anti-KCC2 1:500 and mouse anti-Parv antibody 1:2000 in incubation buffer.

OR

NKCC1+Parv: Dilute rabbit anti-NKCC1 1:500 and mouse anti-Parv antibody 1:2000 in incubation buffer.

OR

Parv Only (for WFA and SYTOX): Dilute mouse anti-Parv antibody 1:2000 in incubation buffer.

Incubate overnight (~24h) at RT, or for 2 days at 4°C. Stir sections occasionally.

3.2 Wash: 3x wash in PBS, 5 minutes.

4.1: Dilute donkey anti-mouse AF488 1:200 and donkey anti-rabbit Cy3 1:200 in incubation buffer. Incubate overnight at 4°C. (for KCC2 and NKCC1)

Dilute donkey anti-mouse Cy3 1:200 and WFA 1:500 in incubation buffer. Incubate overnight at 4°C. (WFA)
Dilute donkey anti-mouse Cy5 1:200 in incubation buffer. Incubate overnight at 4°C. (SYTOX)

4.2 Wash: 3x wash in PBS, 5 minutes.

Store sections in wells with 0.1 M PB and 0.1% sodium azide if mounting later (at 4°C).

For SYTOX only: incubate in 0.05% Triton in PBS for 1 hour
Wash 3x in HBSS, 5 minutes
Incubate in 5uM SYTOX Green in HBSS for 10 minutes
Wash 3x in HBSS, 5 minutes

Sections are mounted on microscope slides from a shallow bath of Tris buffer, then air-dried. Slides are coverslipped in Vectashield mounting medium.

6. Imaging
Open Neuro

Secondary antibody is one conjugated with a fluorophore. The solutions and sections

Double labeling can be done by combining primaries and secondaries (make sure antibody hosts do not overlap) or by sequentially incubating in the primary, followed by secondary, of each.