Supplementary Material

Generation of GPR55 null mutant mice

GPR55 is a G-protein-coupled receptor and is activated by lysophosphatidyl inositol as well as anandamide and Δ^9tetrahydrocannabinol (THC), the latter two under certain conditions (Oka *et al.*, 2007; Waldeck-Weiermair *et al.*, 2008) and thus it may mediate ECS function. GPR55 KO mice were acquired from the Texas Institute of Genomic Medicine (TIGM, Houston, TX). Dr. Andrei Kolovko of TIGM provided the following information on its development: A targeted mutation was generated in 129SvEvBrd-derived embryonic stem (ES) cells using a targeting cassette designed to replace a portion of exon 2 of the *gpr55* gene containing the entire coding region of GPR55 protein with a selection cassette (**Sup. Fig. 1A**). Homologous integration was detected by Southern Analysis using probes internal to the targeting vector on the 5' side and external to the targeting vector on the 3' side **(Sup. Fig. 1A, B)**. Following blastocyst injection, chimeric mice were bred to C57BL/6J mice to generate F1 heterozygous animals. To confirm the loss of GPR55 mRNA in the offspring of these mice, we conducted RT-PCR analysis with GPR55 heterozygous and homozygous KO brain tissues using primers designed to amplify the C-terminal region of GPR55 mRNA. PCR products of the expected size were only detected in cDNAs derived from GPR55 heterozygous brains but not from homozygous brains or mRNA preparations from either genotype **(Sup. Fig. 1C)**. These results indicate that the GPR55 KO mice generated by TIGM are GPR55 null mutants.

GFP in TCA^{mGFP} label the thalamocortical axons originated from the major sensory relay thalamic nuclei

RORα is expressed strongly in the three major thalamic sensory relay nuclei, including the ventrobasal thalamic nuclei (somatosensory), the lateral geniculate nucleus (visual) and the medial geniculate nucleus (auditory) (Nakagawa & O'Leary, 2003). To generate a TCA-GFP line, we generated a double transgenic RORα^{Cre}-Tau^{mGFP} line (named as TCA^{mGFP}) by crossing RORα^{Cre} mice with Tau^{mGFP} mice. RORα^{Cre} mice were generated by inserting an *IRES-cre* expression cassette into the 3' noncoding region of the *RORα* gene. Tau^{mGFP} mice is a Cre-reporter line containing a floxed "stop transcription" sequence in front of membrane anchored GFP (mGFP) and an IRES-NLS-lacZ gene into the Tau locus (Hippenmeyer *et al.*, 2005). This reporter line allows the detection of neurons with Cre-mediated recombination events by the presence of nuclear β-galactosidase (β-gal) and membrane-anchored GFP. At E14.5, GFP in the TCA^{mGFP} brains revealed TCAs extending out from the dorsal thalamus and project ventrally through the ventral thalamus and then turn sharply into the internal capsule (**Sup. Fig. 2B**). At E16.5, TCA-positive fascicles were evident in the striatum and had reached the subplate area of the cortical plate (**Sup. Fig. 2C**). β-gal expression was detected two days after GFP (**Sup. Fig. 2C,D**). This delay relative to GFP was probably due to decreased expression of the second gene secondary to the IRES element. The vast majority of β-gal positive cells were found in the dorsal lateral geniculate nucleus, the ventroposterior nucleus (**Sup. Fig. 2D**), and the medial geniculate nucleus (data not shown), and not in cortex or other area, as expected from RORα expression. Most of the neurons within the ventrobasal thalamus were β-gal positive (**Sup. Fig. 2E**). Postnatally, strong GFP

staining was evident in TCAs passing through the internal capsule, through the striatum, and as they reached primary somatosensory cortex (**Sup. Fig. 2F, G**). Whisker-related GFP clusters were evident in cortical layer IV **(Sup. Fig G, H, I)** and co-localized with VGluT2 (vesicular glutamate transporter 2) **(Sup. Fig. 2I)** immunoreactivity; the latter being a well-known marker for TCA terminals (Fujiyama *et al.*, 2001; Hur & Zaborszky, 2005; Nahmani & Erisir, 2005). This co-localization of VGluT2 and GFP suggests that GFP synthesized in RORα promoter-driven Cre-positive neurons reached TCA arbors within barrels.

To reveal the whisker-related patterns in somatosensory cortex in TCA^{mGFP} mice, the S1 cortex of a P8 TCA^{mGFP} was dissected and flattened following the methods of Strominger and Woolsey (1987). After slight fixation (4% PFA for 30 minutes at room temperature), GFP fluorescence from the TCA^{mGFP} flattened cortex was imaged with an inverted Nikon Eclipse Ti-S epifluorescence microscope (Nikon Instruments Inc.). The GFP pattern reflects whisker-related pattern (**Sup. Fig. 2H**). Strong GFP could also be detected in adult TCA^{mGFP} mice as well as for both the somatosensory and visual pathways (data not shown). In summary, in TCA^{mGFP} mice, TCAs derived from the three major sensory relay nuclei for the somatosensory, visual, and auditory pathways are labeled with strong GFP fluorescence and can be visualized by direct fluorescence. This TCA reporter line should be a valuable tool to identify the location of TCAs for both *in vivo* and *in vitro* experiments.

CTFL antibody targets thalamocortical axons

The CTFL rabbit polyclonal antibody was raised against a GST fusion protein containing the full-length C-terminal sequence of mouse GPR55 protein, KEFRM RIKAH RPSTI KLVNQ DTMVS RG. However, while these antibodies recognized GPR55 (Lauckner *et al.*, 2008) they are not specific for GPR55 as the forebrain CTFL staining pattern in GPR55 KO mouse brain was almost identical to the pattern in their wild type littermates (data not shown). Interestingly, as we were characterizing this antibody, we found it distinctively labeled thalamocortical axonal tracts from E12.5 to P8. Although we do not yet know the epitope detected in TCAs by the CTFL antibody, the strong signal-to-noise ratio of the staining provided by low concentrations of this antibody made it a useful marker for our studies. In addition, CTFL staining also revealed aberrant whisker-related pattern in *barrelless* mutant mice (***adcy1*** null mutant mice (Welker *et al.*, 1996), data not shown). To verify its specificity to detect TCAs, we conducted CTFL and GFP double labeling with TCA^{mGFP} mice at E16.5 and P4 (**Sup. Fig 3**). Almost all GFP^{+}-axon fibers in TCA^{mGFP} are CTFL positive at both ages. In the ventrobasal thalamus, the CTFL-staining pattern resembled the whisker-related pattern known as barreloids and many CTFL-positive fibers projected towards the internal capsule.

Supplementary material and methods

RT-PCR. RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) with a DNAse I step performed to eliminate traces of genomic DNA. cDNA samples were made using SuperScript II reverse transcriptase and random primers (Invitrogen) according to manufacturer's protocol. cDNA synthesis was performed in the presence or absence of

reverse transcriptase in parallel, to control for the possibility of genomic DNA contamination. To detect the expression of GPR55 in mice brain, a primer pair was designed to amplify the c-terminus of GPR55 (forward primer: 5'-CTC CAA CAT CAA CTG CTG CC-3'; reverse primer: 5'-AGC CCA GGA GGG TCT TTA TG-3'). The size of RT-PCR product is 199 bp.

Immunohistochemistry. Tissues were prepared as described in the Materials and Methods in the main text except the thalamocortical slices were prepared as described in (Agmon & Connors, 1991). Primary antibodies (dilution; source): rabbit anti-β-galactosidase polyclonal antibody (1:1000, Millipore, Temecular, CA, AB986), rabbit anti-VGluT2 (vesicular glutamate transporter 2) polyclonal antibody (1:2000; Synaptic System, Göttingen, Germany), and CTFL (1:2000, present study).

Supplementary figure legends

Supplementary figure 1. Generation and validation of GPR55 null mutant mice.

(**A**) Schematic diagram of the targeting strategy for GPR55 shows the genomic structure of the mouse GPR55 gene, the targeting vector, and the locations of the probes used for Genomic Southern Analysis. (**B**) Example of the Southern Analyses used to detect homologous recombination using a 5' internal probe and a 3' external probe (the locations are indicated as boxes on the schematic in **A**). The 5' probe detected an endogenous band of 16.5 kb and homologous targeting creates a band of 20.9 kb in MscI cut genomic DNA. The 3' probe detected an endogenous band of 13.9 kb and homologous targeting creates a band of 15.6 kb in ApaLI cut genomic DNA. **C**: RT-PCR

analysis of brain tissues from GPR55 heterozygous (+/-) and homozygous knockout (-/-) mice. –RT: no reverse transcriptase control.

Supplementary figure 2. GFP in TCA^{mGFP} mice labels thalamocortical axons originating from the primary sensory relay nuclei within thalamus.

(**A**) A schematic view of an embryonic brain slice highlights the path of developing TCAs (red line with arrow head) (**B-I**) TCAs extending toward the cortex are GFP labeled in TCA^{mGFP} reporter mice at E14.5 (**B**), E16.5 (**C-E**), P4 (**F**) and P8 (**G, I**). (**D-E**) β-galactosidase (β-gal) immunoreactivity reveals that Cre activity is primarily restricted to the ventrobasal thalamus (VB) and the dorsal lateral geniculate nucleus (LGN). Many thalamic cells within VB are β-gal positive (**E**). (**G, I**) Thalamocortical slice showing that GFP-TCAs innervate layer IV neurons in the primary somatosensory cortex. (**I2, I4**) GFP-TCAs are co-labeled with VGluT2. (**H**) GFP fluorescence of flattened cortex shows the whisker-related barrel map. Abbreviations: cx, cortex; ge, ganglionic eminence; h, hippocampus; ic, internal capsule; RT, reticular nucleus; st, striatum. B_1-D_1 and α-γ indicates the representation for facial whiskers.

Supplementary figure 3. CTFL immunoreactivity reveals thalamocortical axons.

Double immunostaining shows extensive co-localization of CTFL and GFP immunoreactivity at both E16.5 (**A-D**) and P4 (**F-I**) in TCA-mGFP mouse brains. Abundant CTFL immunoreactivity is present in mGFP-thalamocortical afferents at E16.5 (yellow in **D3**). (**E**) A schematic diagram illustrates the path of thalamocortical axons within a P4 thalamocortical brain slice. (**H**) Confocal images of areas depicted in **F** show

extensive co-localization of CTFL immunoreactivity with GFP. **A**-**C**, **F**, **G** are inverted images of fluorescence images. Abbreviations: cx, cortex; DLG, dorsal lateral geniculate nucleus; ge, ganglionic eminence; ic, internal capsule; RT, reticular nucleus; st, striatum; VLG, ventral lateral geniculate nucleus.