SUPPLEMENTAL DATA

Cognitive deficits in calsyntenin-2 deficient mice associated with reduced GABAergic transmission

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SUPPLEMENTAL MATERIALS AND METHODS

Generation of *Clstn2*$^{-/-}$ Mice

The targeting vector was generated by recombineering (Liu *et al*, 2003) from a 129S7 bMQ BAC clone from the Sanger Institute (Adams *et al*, 2005) and confirmed by sequencing. Linearized targeting vector was electroporated into 129/Ola embryonic stem (ES) cells for homologous recombination (Thomas and Capecchi, 1987). ES cells were selected in the presence of G418 (positive selection) and Ganciclovir (negative selection against random integration). Homologous recombination was verified by Southern blot analysis with probes 5' and 3' to the targeting vector region. Positive clones were injected into C57BL/6J blastocysts, which were transferred to female mice. Founder chimeras were crossed with C57BL/6J mice to obtain germline transmission of the mutation, and resulting germline mutants were backcrossed for at least 9 generations with C57BL/6J mice.

Western Blotting

Brain tissue was collected from P30 *Clstn2*$^{-/-}$ and wild-type (WT) mice and homogenized using a Dounce homogenizer in five volumes of buffer (5 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM DTT, 0.32 M sucrose, supplemented with protease inhibitors). Homogenized tissue was centrifuged at 1000 x g for 10 min at 4 °C and the supernatants were collected. The protein concentrations were determined with a Bio-Rad Protein Assay kit (Sigma) and 20 μg of protein samples were separated by SDS-PAGE on a 10% polyacrylamide gel. Protein transfer was performed onto Immobilon P membranes (Millipore) which were blocked in 5% skim milk in Tris-buffered saline/0.05% Tween-20 and incubated with primary antibodies followed by secondary antibodies. Immunosignals were detected using the SuperSignal Chemiluminescent kit (Thermo Scientific) and visualized using a Bio-Rad gel documentation system.

Elevated Plus Maze

Experiments were conducted in a dimly lit (100 Lux) room as described previously (Clapcote *et al*, 2007). Each mouse was placed in the center of the maze facing a closed arm. Mouse behavior was scored semi automatically using event-recording software (Observer 5.0; Noldus Information Technology). During a 5-min observation, the number of entries (defined as four paws into an arm) and the amount of time spent in open arms, enclosed arms and the central platform were recorded. In addition, the total number of entries into each arm, the number of passages from one enclosed arm to another, the number of head-dips (the mouse extending its head below the open arm), the number of risk assessment (stretch-attend postures) and the number of explorations of the open arm ends (when the mouse was in the final third of an open arm) were recorded.

Forced Swim Test

Each mouse was placed in a transparent plastic cylinder which contained water at 25°C to a depth of 15 cm and forced to swim for 5 min. Floating was defined as immobility, when the mouse ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. Mouse behavior was scored semi-automatically using Observer 5.0 (Noldus Information Technology).

Novel Object Recognition

The novel object recognition test was performed as described (Sik *et al*, 2003) with slight modifications. First, mice were habituated to the novel environment, a clear Perspex arena (41 cm x 41 cm x 33 cm), for 3 days with two 5-min habituation sessions daily. On the fourth day, an acquisition session (15 min) was conducted with two identical objects (glasses; 4 cm diameter x 6 cm height; O1 and O2), placed in two adjacent corners 6 cm from the walls. After a 1 hour interval in the home cage, mice were placed back in the testing arena containing a familiar object (O1) and a new object (laboratory beaker for 10 ml) which replaced O2 at the same location. The time examining each object was recorded for 5 min by EthoVision XT-10 (Noldus Information Technology) and expressed as a percentage of the total time of object exploration. Exploration was defined as directing the nose to the object (distance of < 2 cm). Standing on the object was excluded from the analysis as it is not considered exploratory behavior (Bevins and Besheer, 2006).

Displaced Object Recognition

The displaced object recognition procedure was conducted essentially as described (Ng *et al*, 2009). The procedure consisted of three sessions with inter-session interval 2-3 min, during which mice were returned to their home cage. During the first habituation session (5 min), mice were exposed to the empty arena (41 cm x 41 cm x 33 cm). Next, during the acquisition session (15 min), two identical objects (glasses; 4 cm diameter x 6 cm height) were presented near the corners of the open field. Finally, during the spatial object recognition session (5 min), one object remained in the same location and the second object was moved to the opposite corner (displaced object). The time examining each object was recorded for 5 min by EthoVision XT-10 (Noldus Information Technology) and expressed as a percentage of the total time of object exploration. Exploration was defined as directing the nose to the object (distance of < 2 cm). Standing on the object was excluded from the analysis as it is not considered exploratory behavior (Bevins *et al*, 2006).

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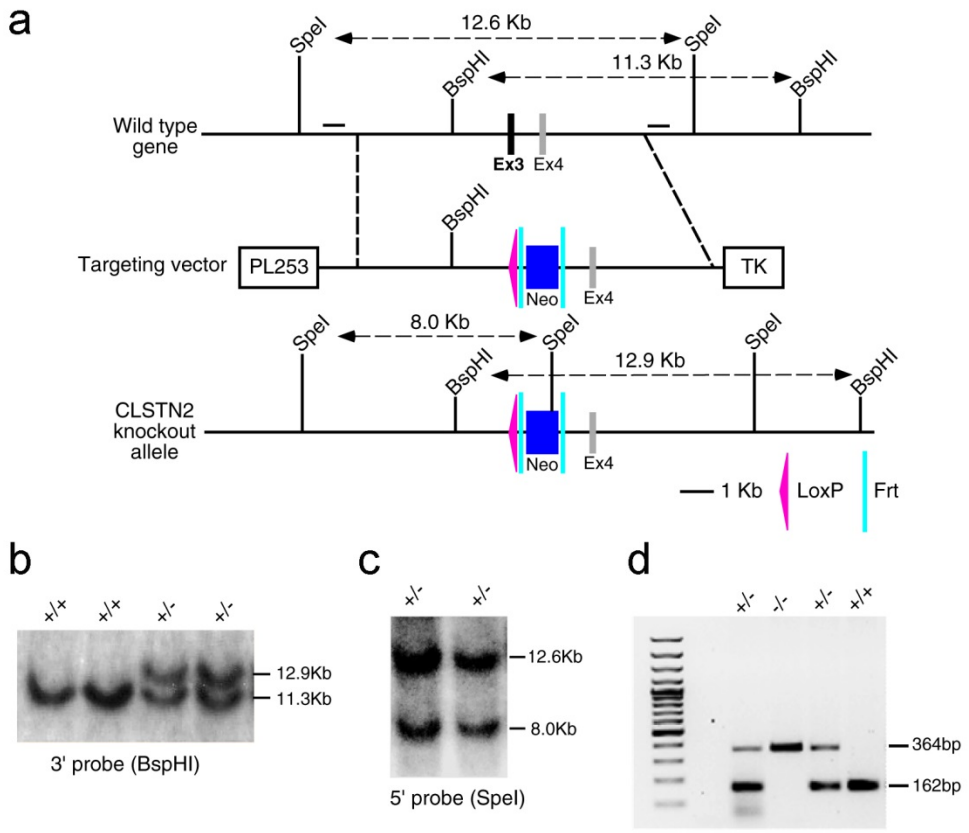

Figure S1. Generation of *Clstn2*$^{-/-}$ mice

(a) Gene targeting strategy. Wild type gene (top) indicates the basic structure of the mouse calsyntenin-2 gene (Ex, exon; selected restriction enzyme sites used for diagnosing homologous recombination are indicated). Probes (black bars) used for Southern blot analysis to monitor homologous recombination are indicated. Targeting vector (center) indicates the structure of the targeting construct introduced into mouse embryonic stem (ES) cells. An additional SpeI site was introduced along with the Neo cassette. TK, thymidine kinase used for negative selection; Neo, neomycin resistance cassette used for positive selection. Knockout allele indicates the structure of the calsyntenin-2 gene after homologous recombination. The 5' 8.0 kb SpeI and 3' 12.9 kb BspHI digestion products assessed by Southern analysis of homologous recombination are indicated. Removal of exon 3 eliminates part of the cadherin domain and alters the reading frame if splicing across the deleted exon occurs.

(b) Southern blot analysis of homologous recombination using a 3' probe. ES cells with a homologous recombination show a 12.9 kb band in addition to the 11.3 kb wild-type band.

(c) Southern blot analysis of homologous recombination using a 5' probe. ES cells with a homologous recombination show an 8.0 kb band in addition to the 12.6 kb wild-type band.

(d) Presence of the knockout allele in mice was diagnosed by a PCR strategy with primers designed around exon 3. PCR of the wild type allele generates a 162 bp band, whereas PCR of a targeted allele generates a 364 bp band.

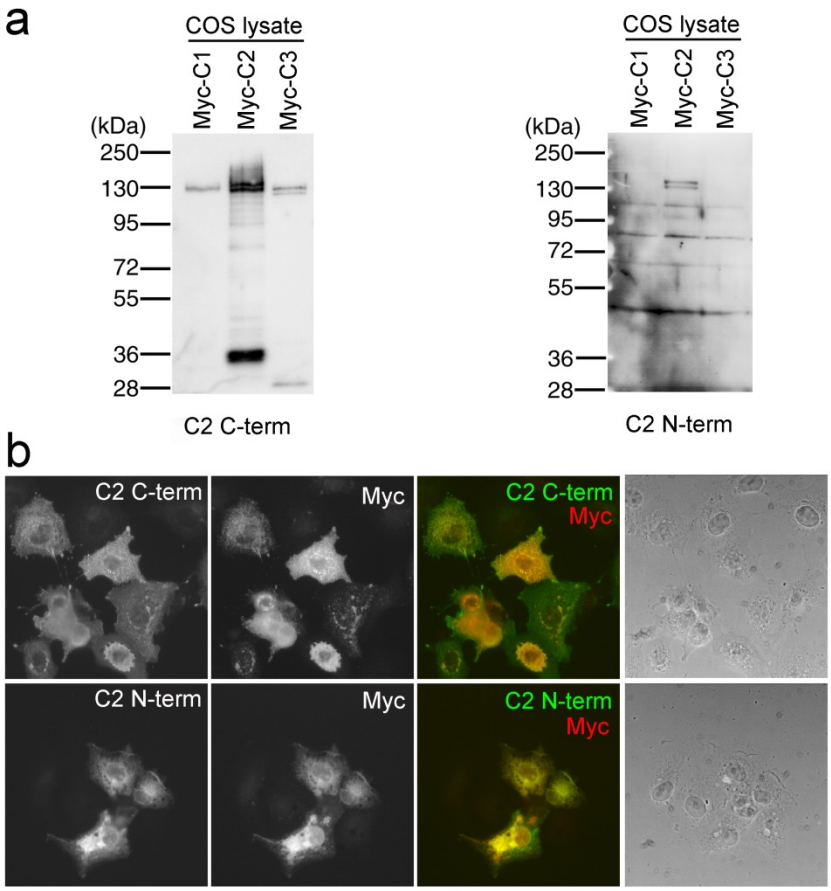

Figure S2. Validation of calsyntenin-2 antibodies

Antisera to the C-terminus and N-terminus of calsyntenin-2 were raised against GST fusion proteins. Antisera were validated by Western blot and immunofluorescence against Myc-tagged calsyntenin-1, -2, or -3 expressed in COS cells.

(a) Western blotting shows that the antiserum to the N-terminus is specific for Myc-calsyntenin-2 (C2; plus some cross-reacting material in COS cells), while the C-terminal antisera shows some cross-reactivity with Myc-calsyntenin-1 (C1) and Myc-calsyntenin-3 (C3).

(b) By immunostaining, both antisera recognize Myc-calsyntenin-2 expressed in COS cells, in a similar pattern as the Myc antibody and with essentially no background immunofluorescence against non-transfected COS cells. However, specific immunoreactivity of cultured neurons or brain slices was not observed, presumably due to limited sensitivity or specificity.