PROTOCOLS

Sacrifice, fixation and processing of the brains for optical microscopy

Mice will be housed in groups in a temperature- and humidity-controlled environment and will be maintained on a 12 h light/dark cycle with free access to food and water. Those animals purchased from an external vendor will be allowed to habituate to our facilities at least one week prior to the start of the experiments. All animal experimentation will be conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the University of Valencia. Every effort will be made to minimize the number of animals used and their suffering.

Animals will be perfused transcardially under deep chloral hydrate anesthesia, first with saline and then with 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4. Thirty minutes after perfusion, the brains will be extracted and cryoprotected with 30% sucrose in PB. Coronal sections (50 μm) were obtained from frozen brains (-30ºC) with a freezing-sliding microtome (Leica SM2000R), collected in 10 (rats) or 6 (mice) subseries and stored at –20 ºC in 30% glycerol, 30% ethylene glycol in PB 0.1M until used. In some cases in which the morphology of fine structures such as spines was not clearly identified, we will cut directly the brains with a vibratome and will keep the sections in PB with 0,01% sodium azide until use.

Conventional immunohistochemistry for optical microscopy

Tissue will be processed "free-floating" for immunohistochemistry as follows. In the case of PSA-NCAM immunohistochemistry, sections will be previously incubated for 1 minute in an antigen unmasking solution (0.01 M citrate buffer, pH 6) at 100ºC. After cooling down the sections to room temperature, they will be incubated with 10% methanol, 3% H2O2 in phosphate buffered saline (PBS) for 10 minutes to block endogenous peroxidase activity. After this, sections will be treated for 1 hour with 5% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 0.2% Triton-X100 (Sigma-Aldrich, St. Louis, MO) and will be incubated overnight at room temperature (or 48 hours at 4ºC) in primary antibodies (examples: monoclonal mouse Men-B anti-PSA-NCAM antibody (1:1400; Abcys, France), monoclonal mouse anti-synaptophysin antibody (1:200; Sigma-Aldrich), monoclonal mouse anti-GAD67 antibody (1:500; Developmental Studies Hybridoma Bank, Ames, IA) or monoclonal mouse anti-TH antibody (1:200; Chemicon Int. Inc., Temecula, CA)). After washing, sections will be incubated for 2 hours with donkey generated biotinilated secondary antibodies (1:250; Jackson ImmunoResearch Laboratories), followed by an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) for 1 hour in PBS. Color development will be achieved by incubating with 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and H2O2 for 4 minutes. PBS containing 0.2% Triton-X100 and 3% NDS will be used for primary and secondary antibodies dilution. All of the sections studied will pass through all procedures simultaneously, to minimize any difference from immunohistochemical staining itself. Appropriate controls for immunohistochemistry will be we performed in each case, by preincubation with the antigenic peptide/carbohydrate, by omission of primary antibodies and their substitution by normal donkey serum.

Single, double and triple fluorescence immunohistochemistry

In general, sections will be processed as described above, omitting the endogenous peroxidase block. The first day, sections will be incubated overnight at room temperature with the primary antibody cocktails from different species. Then, sections will be washed and incubated for 1 hour in different fluorescent secondary antibodies directed to the species of primary antibodies.

Confocal microscopy and analysis of the data

All sections processed for fluorescent immunohistochemistry will be mounted on slides and coverslipped using fluorescent water-based mounting medium. Then, the sections will be observed under a confocal microscope (Leica SPE). Z-series of optical sections (1 μm apart) will be obtained using sequential scanning mode. These stacks will be processed with LSM 5 Image Browser software.

Sacrifice, fixation and processing of the brains for transmission electron microscopy

Mice processed for electron microscopy will be perfused transcardially under deep chloral hydrate anesthesia, first with saline for 1 min, followed by 450 ml solution of paraformaldehyde 2% in a lysine-phosphate buffer, pH 7.4. The lysine-phosphate buffer was prepared 1:1 from a solution of PB 0.1 M pH 7.4 and a solution 0.2 M of lysine adjusted to pH 7.4 using a solution of Na2HPO4 0.1 M. The buffer is mixed with a concentrated solution of paraformaldehyde 3:1 and 0.214 g of sodium peryodate is added for each 100 ml just before use. Brains will be extracted and sliced with a vibratome at 50 or 100 um as described above.

GFP immunohistochemistry for electron microscopy

The sections destined for electron microscopy will be cryoprotected for 30 minutes by immersion in 25% sucrose and 10% glycerol in 0.01 M PB and then will undergo freeze–thawing three times with liquid nitrogen to enhance antibody penetration. Then, sections will be washed in PB and the endogenous peroxidase activity will be blocked as described above, without methanol. Subsequently, non-specific binding sites will be blocked with NDS 10% in PB with glycine 0.2% and lysine 0.2%. After washing, sections will be incubated 48 hours at 4 ºC in polyclonal chicken against GFP (1:1000, Millipore). After washing, sections will be incubated for 2 hours with donkey generated biotinilated secondary antibodies (1:250; Jackson ImmunoResearch Laboratories), followed by an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) for 1 hour in PBS. Color development will be achieved by incubating with 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) and H2O. Immunolabeling will be intensified treating the sections with 1% osmium tetroxide (EMS, Hatfield, PA) in PB for 60 min, protected from light. After each step, sections were carefully rinsed in PB.

Inclusion and ultramicrotomy

All the sections processed for electron microscopy will be stained with uranyl acetate 1% in ethanol 70% for 1 hour at 20 °C, dehydrated through increasing graded ethanol series, cleared in propylene oxide and flat-embedded in Durcupan. After analysis of this material under the light microscope, selected PSA-NCAM expressing interneurons from the sections will be re-embedded in Durcupan and 60-nm thick ultrathin sections will be cut with an ultramicrotome (Leica, EM-UC6). Ultrathin sections will be serially collected on formvar-coated single-slot copper (DAB labeling) or nickel grids (gold labeling) and stained using lead citrate. Selected cells will be observed and partially reconstructed from serial sections under a JEOL JEM-1010 electron microscope.

Quantification of dendritic arborization, spine density and spine morphology

The analysis of dendritic arborization ad spine density will be performed in GAD-GFP expressing interneurons. These parameters will be studied using confocal microscopy (Leica SPE). Z-series of optical sections (0.2 µm apart) covering the complete dendritic tree of selected interneurons. The stacks obtained will be then processed using ImageJ software (NIH) in order to render 3D reconstructions in which we could analyze the exact distance of the branching and terminal points of the dendrites of a given interneuron. The degree of dendritic arborization will be analyzed using a procedure deriving from the Sholl profile. The Sholl analysis consists of measure the number of intersections with circles of increasing radius centered in the soma. With this method the only information needed is the distance of both the terminal points of dendrites and the branching points. To study the spine density of interneurons we selected individual dendrites from GFP-expressing neurons. Stacks of confocal images were obtained with the 63x objective and an additional 3.5 digital zoom. The spines were counted in three dendritic fragments (60 µm each) expanding to 180 µm from the soma. The morphology of spines will be studied as described before (Kawaguchi et al., 2006), measuring spine neck length and distinguishing between thin, stubby, multiple head and mushroom spines, filopodia, and fan-like processes.

Preparation of organotypic cultures

Organotypic slice cultures will be prepared from 8-days-old transgenic GAD-GFP mice. Mice pups will be decapitated and their brains will be removed from the skull under sterile conditions and placed into Petri dishes filled with cold (4º C) sterile dissecting medium [Gey's Balanced Salt Solution (GBSS, Life Technologies, Gaithersburg, MD), glucose (MERCK), penicillin/streptomycin, hexamycin, L-glutamin, fungizone (GIBCO)]. The overlying pia will be gently removed and coronal cuts will be made to remove portions of the rostral and caudal poles, leaving the frontoparietal region intact. The right and left cortices will be cut simultaneously in the coronal plane at a thickness of 350 mm with a McIlwain tissue chopper. The slices will be transferred into dissecting medium and separated gently by agitation with a fire-polished glass pipette. Slices containing mPFC will be placed on moistened translucent membranes of tissue culture inserts (0.4 μm, Millicell-CM, Millipore, Bedford, MA, USA) immersed in Serum-OPTIMEM culture medium (50% MEM; 25% Hank's balanced salt solution; 25% heat inactivated horse serum; Gibco BRL, Life Technologies, Paisley, Scotland; supplemented with glutamine to a final concentration of 2mM and 0.044% sodium bicarbonate, $NaHCO_3$). Three or four slices will be cultured in the same insert and four inserts will be placed together in a single six-well plate and designated as a control or treatment group to ensure that the 18–24 slices in each group will be cultured under identical conditions. The control and treatment groups for a single trial will be obtained from the same litter, prepared at the same time, and cultured for the same durations. Cultures will be stored in a humid atmosphere at 35°C, in 5% CO_2 and incubated for 14 days. Medium will be changed every 48 hours. A similar procedure will be used for the cultures of hippocampal slices, dissecting the hippocampus and obtaining transverse sections (Stoppini et al., 1991).

Western blot analysis

Brains will be removed after decapitation and cooled on ice, and the mPFC will be dissected out. Samples will be immediately frozen in liquid nitrogen and kept at -80ºC until use. For protein extraction, tissue will be homogenized in 1% NP40, 0.1% SDS, 0.5% Sodium deoxycholate , 150 mM NaCl and 50 mM Tris-HCl (pH 8.0) containing a mixture of protease inhibitors (Sigma). Samples will be kept in rotation at 4ºC for 2 hours and then, centrifugated at 12000 rpm and 4ºC for 20 minutes. Supernatant will be assessed for the amount of protein using Bradford reagent (Sigma) at 595 nm with BSA as the standard. Protein extract will be boiled for 1 minute in reducing electrophoresis buffer and then, samples containing 20 μg of protein were resolved on 7,5% or 10% (depending on the protein analyzed) SDS-polyacrylamide gels at 32 mA, 250 V for 2 hours. Prestained broad-range protein ladders (Fermentas International Inc, Canada, USA) will be included to measure molecular weight of individual bands. Protein extracts (at different empirically tested quantitities) of protein/lane, will be separated on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes using an electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes will be stained with Ponceau S to confirm equal loading and transfer of the gels. Subsequently, the membranes will be cut into two parts, and the lower part will be taken to determine β-actin protein levels. The blots will then be incubated overnight at 4 °C with different primary antibodies and monoclonal mouse anti-β-actin (1:5000, Sigma). Immune complexes will be detected using appropriate peroxidase-conjugated secondary antibodies. Immunoreactive bands will be detected with the enhanced chemiluminescence system, ECL (Amersham Pharmacia, Piscataway, NJ, USA) exposing later the blots to Kodak XAR-5 film (Sigma). The relative levels of immunoreactivity will be quantified using Image Gauge (Fujifilm) and Image-Pro Plus (Media Cybernetics Inc, Bethesda, MD, USA) software (available in the Cell Biology Department and the Central Research Support Services of our campus). To normalize for small variations in loading and transfer, the ratio of proteins level to β-actin will be calculated for each sample. Molecular weights of immunoreactive (IR) bands will be calculated on the basis of the migration of molecular weight markers (Roche) using Image Gauge (Fujifilm) software.

Quantitative RetroTranscription-Polymerase Chain Reaction

Mice used for qRT-PCR were sacrificed by decapitation using a guillotine. After that, brains were removed from the skull and the different regions to be analysed were extracted. Total mRNA was extracted using TriPure reagent (Roche Applied Science, Indianapolis, IN) following manufacturer's instructions. The concentration and purity of total RNA was determined by Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). cDNA synthesis was performed using the Expand reverse transcriptase (Roche Applied Science).

Specific primers to mouse *GAD67, GAD65, NCAM, ST8SIAII, ST8SIAIV , SYN, VGLUT1, VGAT, ERB4, BDNF, GABAA,..* have been already designed in our lab from public sequences, which were obtained from Ensembl Genome Browser data base (http://www.ensembl.org/) using PrimerBlast free software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed between exons to avoid DNA contamination, when possible. The primer sequence for the reference gene (*ywhaz*) was obtained from Bonefeld et al. (2008). Primers were tested for nonspecific products and correct amplicon size by electrophoresis in 1,5% EtBr agarose gel. qPCR was carried out in triplicates with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems), primers at a concentration of 240 nM, and 4 μl of cDNA (25 ng/μl). Following a 95 °C denaturation for 10 minutes, the reactions were cycled 40 times with a 95 º C denaturation for 15 seconds and a 60 º C annealing step for 1 minute. After this, a melt curve was performed to assess the specificity of primers. Relative quantification was performed using the comparative threshold (Ct) method according to the $2^{-\Delta\Delta Ct}$ method. Changes in gene expression were reported as fold changes relative to controls. An unpaired *t*-test was performed to analyze the statistical significance of results.

Analysis of single confocal planes of puncta

We will analyze the density of puncta expressing different markers in focal planes of different regions (hippocampus and different regions of the neocortex). Confocal z-stacks covering the whole depth of the sections will be taken with 1 µm step size and only subsets of confocal planes with the optimal penetration level for each antibody were selected. On these planes, small regions of the neuropil (505 µm2) will be selected for analysis, in order to avoid blood vessels and cell somata. Images will be processed using ImageJ software as follows: the background will be subtracted with rolling value of 50, converted to 8-bit deep images and binarized using a determined threshold value. This value depended on the marker and the area analyzed and will be kept the same for all images with the same marker and area.

Then, the images will be processed with a blur filter to reduce noise and separate closely apposed puncta. Finally, the number of the resulting dots per region will be counted. Means will be determined for each animal group and the data will be subjected to two-way ANOVAs with repeated measures, followed by Bonferroni post hoc tests.

Analysis of the distribution of F-Actin and IP3K

After perfusion and sectioning of the brains with a vibratome (150 µm), we will perform immunohistochemistry for GFP, F-actin and IP3K. Then, using a simple blind scoring system IP3K-positive and actin-positive GFP expressing interneurons in the sections will be examined on a confocal microscope and categorized as punctate, intermediate (still punctate, but with spine fluorescence equal to or less than the shaft) or striated, having the majority of the immunoreactivity in the dendritic shaft. Sets of 18–30 slides from DOI treated animals or controls will be assigned random numbers by a blind investigator. Then, for each slide in the set, between 50 and 300 IP3K/actin-positive neurons from randomly selected areas on the slide will be classified into one of the three categories (Schell et al., 2006).

Behavioral characterization

Hole board test

Each mouse will be placed in the centre of the hole board (21 X 21·X 36 cm transparent Perspex chamber with non-transparent floor raised 5 cm above the bottom of the chamber with 12 equally spaced holes, 2 cm in diameter) and will be allowed to explore the chamber for 3 min. The distance travelled and number of holes explored (= head dips) will be monitored by two layers of infrared photo beams connected to a computer equipped with the 'ANY-maze' software (Stoelting Europe).

Prepulse inhibition test

To measure the startle reactivity, mice will be placed in small metal cages (90 X 40 X 40 mm) which restrict major movements and exploratory behaviour. The cages are equipped with a movable platform floor attached to a sensor recording vertical movements of the floor. The cages are placed in four sound-attenuating isolation cabinets (TSE). A startle reflex will be evoked by acoustic stimuli delivered from a loudspeaker suspended above the cage and connected to an acoustic generator. The startle reaction of a mouse to the acoustic stimuli evokes a movement of the platform. The transient force resulting from this movement of the platform will be recorded on a personal computer during a recording window of 260 ms and stored in the computer for further evaluation. The recording window will be measured from the onset of the acoustic stimuli. An experimental session will consist of a 2 min habituation to a 65 dB background white noise (continuous throughout the session), followed by a baseline recording for 1 min at background noise. After baseline recording, 6 pulse alone trials using the startle stimuli of 120 dB intensity and 40 ms duration will be applied in order to decrease influence of within session habituation. These data will not be included in the analysis of the prepulse inhibition. For tests of prepulse inhibition, the 120 dB/40 ms startle pulse will be applied either alone or preceded by a prepulse stimulus of 70, 75 and 80 dB intensity and 20 ms duration. An interval of 100 ms with background white noise will be employed between each prepulse and pulse stimulus. The trials will be presented in a pseudorandom order with an interval ranging from 8 to 22 s. Amplitude of the startle response (expressed in mV) will be defined as a difference between the maximum force detected during a recording window and the force measured immediately before the stimulus onset. Amplitudes will be averaged for each individual animal, separately for both types of trials (stimulus alone, stimulus preceded by a prepulse). Prepulse inhibition will be calculated as a percentage of the startle response using the formula: % prepulse inhibition = 100 -- [(startle amplitude after prepulse -- pulse pair)/ (startle amplitude after pulse only) X 100].

Cranial window surgery**.**

Cranial windows will be bilaterally implanted over the somatosensory cortices of GIN mice as described previously (Holtmaat et al., 2009). Initially we will perform this procedure on a location above the somatosensory cortex. If the development of the project allows it, we will begin to perform this procedure on the prefrontal cortex, which is a more complicated region to perform surgery due to its more restricted area, the thickness of the bone and the abundant presence of vascularization. Mice will be anesthetized with xylazine-ketamine (0.1 mg/g bw & 0.01mg/g bw respectively) and monitored by breathing rate and foot pinch reflex. Skin over the dorsal surface of the skull will be shaved, the head positioned in a stereotaxic frame, and eye lubricant applied to prevent ocular dryness. The shaved area will be cleaned with povidone-iodine (Betadine) and isopropyl alcohol. Surgery will be carried out under a surgical dissection scope following a rigid aseptic technique. A 3-cm midsagittal incision will be made over the parietal and occipital portions of the skull. Soft tissues will be reflected laterally by blunt dissection and retracted by hemostats. The pericranium will gently scraped from the skull with a scalpel. Using stereotaxic coordinates, somatosensopry cortices will be located and 5-mm diameter circles drawn with a pencil over the target areas.

The skull will be thinned along the drawn circles with a fine drill using a 0.5-mm diameter round burr. To avoid thermal injury and wash away bone dust, the drilling field will be frequently washed with sterile saline. Impending penetration will be frequently assayed by careful pressure with a fine probe. Penetration of the bone is usualy indicated indicated by the appearance of clear fluid along the bottom of the drilled groove. When the bone flap yielded to careful pressure along most of the circle, the bone flap will be carefully removed with fine forceps leaving behind the dura.

Debris will cleared and 5-mm diameter circular glass cover slips (#1, EMS) will be positioned over the openings. The external bone edges of the craniotomies will be checked for dryness and histocompatible cyanoacrylate glue (VetBond, 3M) and dental cement (Stoelting) will be applied over the juncture between coverslip and bone as firm pressure is applied to keep the cover slips in place. A few minutes were given for the glue and the bone cement to begin setting. Then the scalp tissue along the incision will be adhered to the periphery or the cranial window with cyanoacrylate glue, leaving the bilateral cranial windows exposed. Finally, the mouse will be given lactated ringers solution (SC, 0.015 ml/g) and the analgesic, Buprenex (SC, 0.3 mg/ml 2_ daily for 5 days), and then returned to its cage for recovery under observation with sulfamethoxazole (1 mg/ml) and trimethoprim (0.2 mg/ml) chronically administered in its drinking water through the final imaging session.

Two-Photon Imaging and Data Analysis.

Starting at three weeks after surgery, windows will be scanned for optical clarity and only clear windows will be used for imaging. Young adult mice (12 weeks postnatal) previously implanted with cranial windows were anesthetized with xylazine-ketamine (0.1 mg/g bw & 0.01mg/g bw respectively) or isoflurane. Anaesthesia will be monitored by breathing rate and foot pinch reflex and additional doses of anaesthetic were administered during the imaging session as needed. *In vivo* two-photon imaging wil be performed in an Olympus Fluoview FV1000 upright multiphoton microscope and acquisition software (Olympus/Imaris) for *in vivo* imaging (http://scsie.uv.es/scsie_new/web_cod/SCSIE/MIC/es/MIC_equipos_es.php).

The animal will be placed on a custom-made stereotaxic restraint with controlled temperature affixed to the pletina (Supletech instruments Inc). The head will be positioned in the stereotaxic restraint and nine slightly overlapping volumes in a 3 X 3 array will be imaged through *z-x-y* translation of the motorized stage (*z*-spacing 1.5 microns). The light source for two-photon excitation is a commercial

Mai-Tai HP DeepSee (Spectra Physics). The excitation wavelength will be set to 900 nm, with the excitation signal passing through a 25X/1.05 NA water immersion objective with a working distance of 2mm (Olympus) and collected after a barrier filter by a photomultiplier tube.

Generation of "double-hit" model of schizophrenia in mice

This model combines a pharmacological treatment with a social stressor. Mice were injected with the NMDA antagonist MK-801 (1mg/kg) at P7. After weaning (21 days) male rats were placed in individual cages and reared in isolation individually. All mice will be housed in the same room, and sharing the same light, temperature and humidity. Mice reared in isolation could hear and smell the other mice, but were unable to see or have physical contact with them. All animals were handled once a week by the same person, who replaced the bedding of the cage and added food and water. Animals remain isolated until adulthood and during the treatment with haloperidol.