Analysis of Perisomatic Innervation in Organotypic Slice

Immunohistochemistry, imaging and analysis: Slices were fixed, freeze-thawed and immunostained as described (Chattopadhyaya et al., 2004). Primary antibodies were used at following concentrations: NeuN (mouse monoclonal, 1:400, Chemicon), Parvalbumin (rabbit polyclonal, 1:1000, Sigma), GABA (rabbit polyclonal, 1:1000, Sigma). Confocal imaging and data analysis were performed as described (Chattopadhyaya et al., 2004). To quantify the colocalization between anatomically identified varicosities and syn-GFP or immunopositive-GAD65 puncta, slices were co-transfected with, respectively, P_{G67}-tdtomato and P_{G67}-syn-*GFP* or P_{G67}-*GFP* only followed by immunostaining with GAD65 antibody (mouse monoclonal, 1:1000, Chemicon). Basket cells were imaged 6 days after transfection. The green and red channels were separated, then images were thresholded and puncta were counted automatically using ImageJ software. Particles ranging from 0.15-2.5 μm^2 were quantified. The green and red channels were then combined and only the syn-GFP or GAD65 puncta that were localized along the axon of transfected basket cell were quantified.

The effect of activity blockade on GABA expression was evaluated in TTX-treated and control age-matched cultures by counting the number of GABA and NeuN-immunopositive cells in confocal stacks acquired from at least 4 organotypic slices for each experiment. This experiment was repeated three times. The intensity of somatic GABA signal was measured using ImageJ. Care was taken to use the same laser setting for all the acquisitions.

Analysis of Local Axon Arbor and Innervation Field of Basket Interneurons

Confocal image stacks of basket cell axon arbors were acquired using a 63X oil objective (Zeiss NA 1.4) with 1-μm steps of at least 25 μm along the z-axis. To reconstruct basket cell dendritic and axon arbors in Figure 5A, overlapping image stacks were acquired to cover the whole dendritic field and most of the axon arbor. Image stacks were merged together and traced using the confocal module of Neurolucida (Microbrightfield). To quantify innervation field in Figure 5B, at least 2 image stacks from regions with the highest axon density were taken for each basket cell within 300 μm radius from the basket cell soma. Image stacks were traced using the Neurolucida confocal module and analyzed using Neuroexplorer (Microbrightfield). Basket cell dendrites, axons, boutons, and pyramidal cell

somata were traced in image stacks. The following parameters were analyzed and reported: (1) axon density, computed as total axon length (μm) in a unit volume (1000μm^3); (2) number of basket axon branches that contact each pyramidal cell soma; (3) average interbranch interval, computed as axon length (in μm) between 2 neighboring branch points; (4) percentage of basket boutons on pyramidal cell somata, computed as ratio of boutons on pyramidal cell somata to the total number of boutons in the stack volume; (5) percentage of targeted pyramidal cell somata, also defined as target coverage for the basket cell, computed as ratio of number of pyramidal cell somata contacted by at least one axon branch and bouton to the total number of pyramidal somata in the stack volume. For each parameter, average value ± SEM is reported.

Analysis of Perisomatic Innervation in Visual Cortex of *Gad1* $^{lox/lox}$ Mice Injected with *AAV-GFP-ires-Cre*

Tissue processing: At P20 or P32, AAV-injected mice (P13) were anesthetized (sodium pentobarbitone, 6 mg/100 g body weight) and perfused transcardially with 4% paraformaldehyde in phosphate buffer (pH 7.4). Coronal sections (80 μm) were cut from visual cortex using a vibratome (Leica VT100). Brain sections were blocked in 10% NGS (Normal Goat Serum) and 1% Triton X-100. Slices were then immunostained with anti-NeuN (monoclonal, 1:400, Chemicon), and anti-parvalbumin (Pv) antibody (rabbit 1:1000, Sigma) for analysis of bouton size, or anti-GABA (rabbit 1:1000, Sigma) and Pv (mouse 1:1000, Sigma) for analysis of GABA level followed by Alexa594-conjugated anti-rabbit IgG and Alexa633-conjugated anti-mouse IgG (Molecular Probes, 1: 400) and mounted in Vectashield mounting medium (Vector).

Image acquisition and analysis: Confocal images (Zeiss LSM510) were taken using a 63X oil objective (Zeiss NA 1.4). Z-stacks were acquired with 0.7-μm steps. Scans from each channel were collected in multi-track mode and subsequently merged. Care was taken to use the lowest laser power and no bleed through was visible between channels. Bouton size of GFP-positive boutons co-expressing parvalbumin was measured by the bouton diameter perpendicular to the basket axon around pyramidal cell somata using Zeiss confocal software. All quantification was done blind to genotype.

Analysis of Perisomatic Innervation in Visual Cortex of Germ-line *Gad1* $^{+/-}$ Mice.
At P28, *Gad1* $^{+/-}$ mice and their *Gad*$^{+/+}$ littermates were anesthetized and perfused transcardially as described above. Coronal sections (80 μm) were cut from visual cortex using a vibratome (Leica VT100). Brain sections were blocked in 10% NGS (Normal Goat Serum) and 1% Triton X-100. Slices were then immunostained with anti-GAD65 (monoclonal, 1:1000, Chemicon), for analysis of perisomatic innervation, or with anti-GAD65 and anti-parvalbumin (Pv) antibody (rabbit 1:1000, Sigma) for colocalization studies, followed by Alexa488-conjugated anti-mouse IgG and Alexa594-conjugated anti-rabbit IgG (Molecular Probes, 1: 400) and mounted in Vectashield mounting medium (Vector). Confocal images (Zeiss LSM510) were taken using a 63X oil objective (Zeiss NA 1.4). Three coronal sections containing the primary visual cortex from each animal were analyzed. Images were saved as TIFF files and analyzed with ImageJ software. Neuronal somata (>80% are pyramidal cell somata) appear as large GAD65 negative profiles (Figure 7E). To quantify perisomatic GAD65 puncta, an area 2 μm distal from the edge of a neuron profile was traced and outlined. GAD65 puncta within this area were counted automatically using ImageJ software as puncta density -- the number of GAD65 puncta per cell soma section. For each animal, puncta densities from 25 or more neuron soma sections were averaged. The mean values of puncta density for each animal were used to generate bar graphs and SEM. All quantification was done blind to genotype.

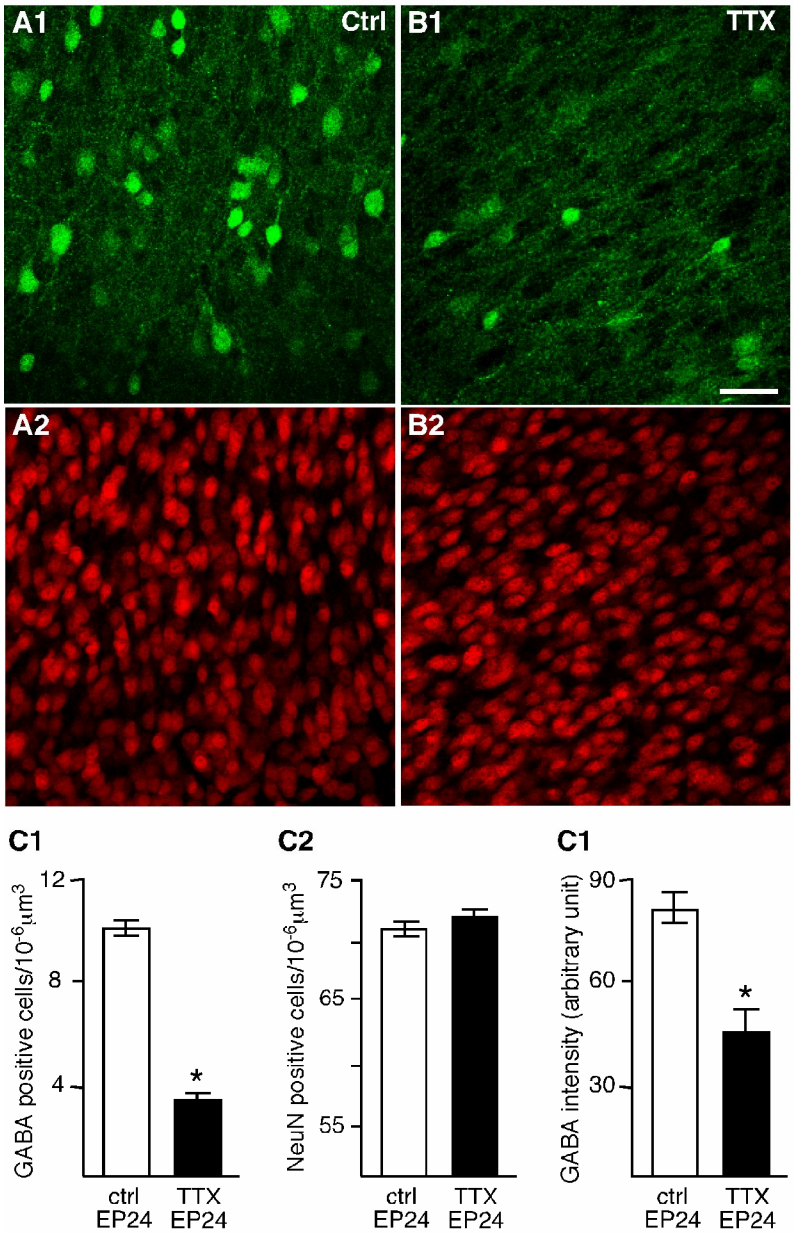
Western Blotting
GAD67 and GAD65 protein levels were quantified using 3 *Gad1*$^{+/-}$ vs 3 *Gad1*$^{+/+}$ mice. Protein lysates were prepared by homogenizing tissue from both somatosensory and visual cortex of P28 mice in 50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1%NP-40, 0.1%NaDeoxycholate, 10mM NaF, 1mM NaOrthovanadate, 1X protease inhibitor cocktail (Roche). Protein levels were quantified using Bicinchoninic Acid (BCA) Protein Assay reagents Kit (Pierce, cat#23228) and equal amounts were loaded in each lane. Proteins were separated on pre-cast 7.5% polyacrylamide gels (Biorad) and transferred onto Millipore Immobilon-P membrane, which were blocked by incubation in PBS with 5% dried milk and 0.05% Tween-20. Membranes were probed with the following primary antibodies: anti-

GAD67 1:1000 (mouse monoclonal, Santa Cruz), anti-GAD65 1:1000 (mouse monoclonal, Chemicon), anti-ßactin 1:14000 (mouse monoclonal, Sigma). HRP-conjugated anti-mouse secondary antibody (Pierce) was then added to the blots. Immuno-reactive bands were detected with chemiluminescence reagents (Pierce Super Signal Femto Substrate) and signal visualized by exposing membrane to Iso-Max autoradiography/X-ray film (Sci-Mart). To normalize levels of GAD67 and GAD65 to ß-actin, non-saturating exposure of western blots were scanned and bands intensities were analyzed using ImageJ software. Three *Gad1*$^{+/-}$ and three *Gad1*$^{+/+}$ mice were used for quantitative analysis.

Immuno Electron Microscopy

Organotypic culture were immersed in a solution of 0.2% gluteraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (4°C, pH 7.4) and microwaved for 8secs. Slices were then cryoprotected and freeze thawed in liquid nitrogen. These were then incubated overnight in the primary antibody (GFP 1:600, Chemicon) in PBS at 4°C, then for 2 hours at room temperature in biotinylated secondary antibody (1:500 goat antirabbit (F)ab fragment, Jackson Laboratories, USA). To reveal the labeling, avidin biotin peroxidase complex (ABC Elite, Vector Laboratories, USA) was used for 2 hours, and incubated in 3, 3'-diaminobenzidine tetrachloride (Fluka, Switzerland) and 0.015% hydrogen peroxide. Following enhancement, the sections were then washed, postfixed in osmium tetroxide and embedded in Epon resin (Fluka). Once cured the regions of interest were localized with the light microscope and this area cut away from the section. This was then attached to a blank resin block and serially thin sectioned at a thickness of between 50-60nm. Images were acquired with a Philips CM12 electron microscope with a filament voltage of 80kV using a digital camera (Megaview 3, SIS, Germany).

Supplementary figure 1

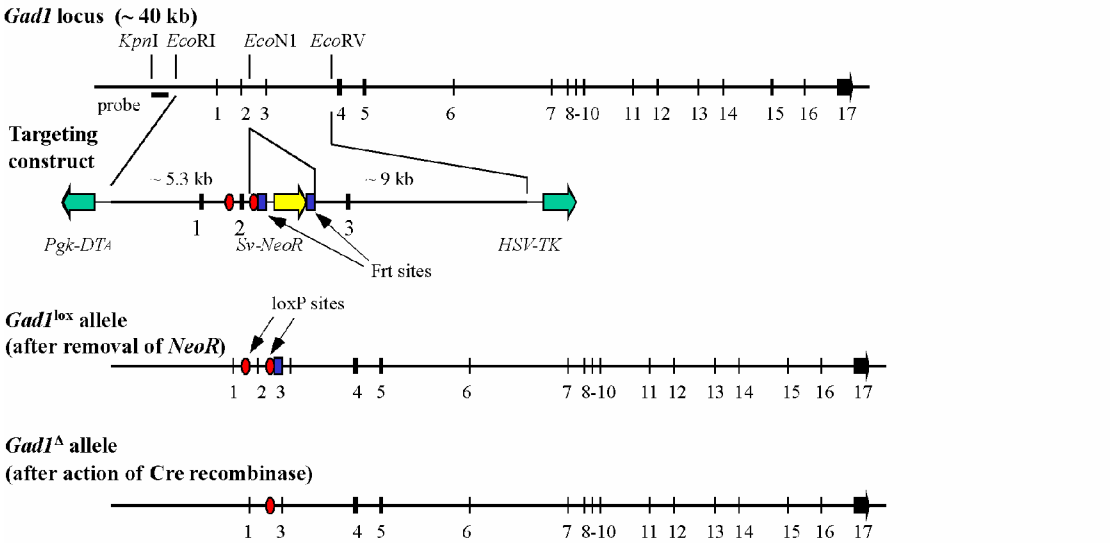


Supplementary Figure 1. Blockade of spiking activity reduces GABA levels in cortical organotypic cultures.

The number of GABA-immunopositive cell somata (green) is strongly decreased in TTX-treated cortical slice cultures from EP18-24 (**B1**) as compared to control untreated slices

(**A1**). This reduction is not due to neuronal cell death because the density of NeuN-positive profile (red) in unaffected by TTX application (**A2**, **B2**). Scale bar: 50 μm. **C**, Quantification shows that the density of GABA-positive cell somata is reduced by 57% in TTX-treated cortical slices (**C1**; n=9 slices for both treated and untreated group, t-test $p<0.01$) while in the same slices the density of NeuN-positive cells in unaffected (**C2**; t-test $p>0.1$). The mean intensity of the somatic GABA signal is reduced by ~42% in TTX-treated slices (**C3**, n=51 cells from TTX-treated slices, n=87 cells from control untreated slices; Mann-Whitney U test, $p<0.001$

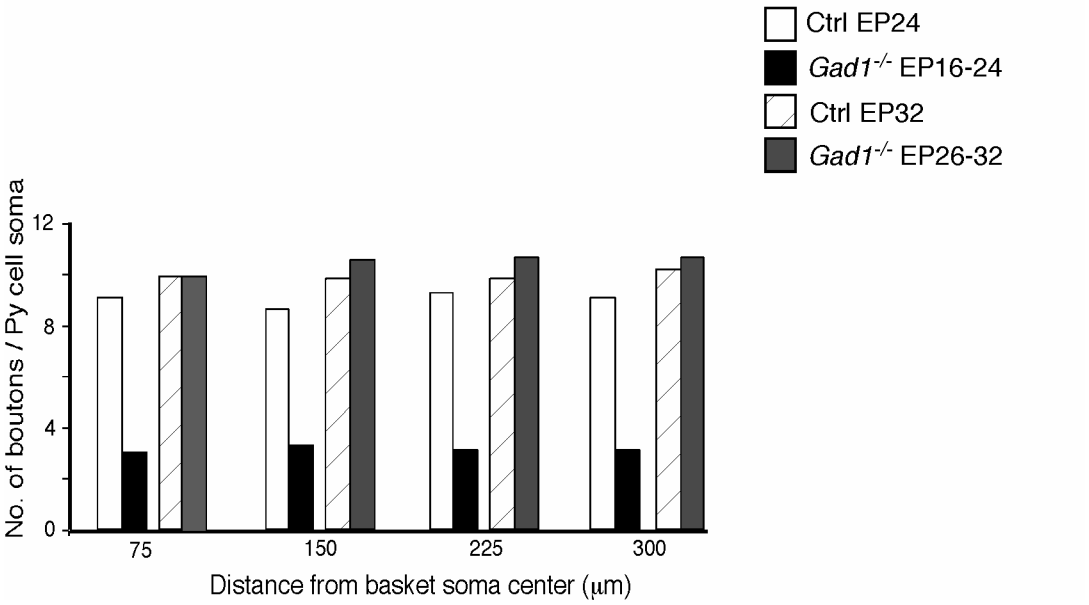
Supplementary figure 2



Supplementary Figure 2. Schematic showing the generation of *Gad1*$^{lox/lox}$ allele

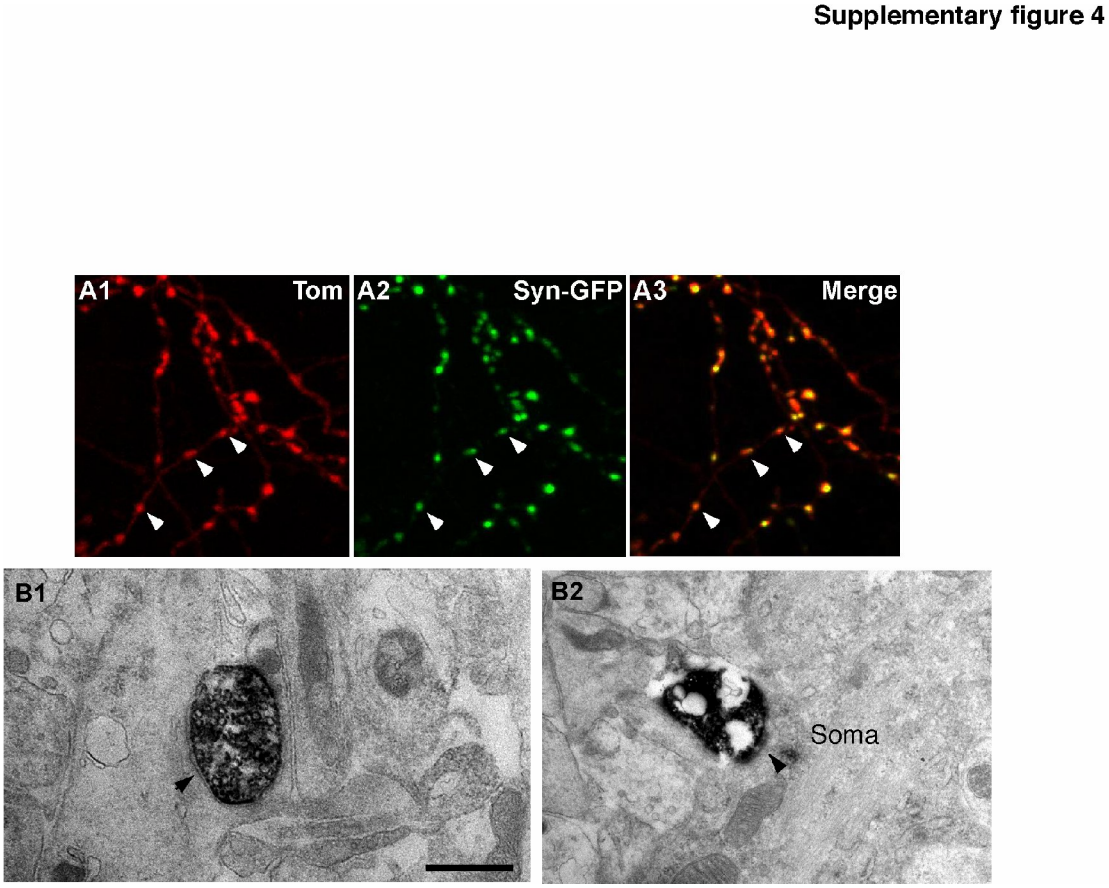
To generate *Gad1*$^{lox/lox}$ mice, exon 2 (the first coding exon) of the *Gad1* gene was flanked by loxP sites using gene targeting in ES cells. After removing the Sv-NeoR selectable gene by the action of FLP recombinase, the resulting *Gad1*$^{lox/+}$ mice were bred with each other to generate *Gad1*$^{lox/lox}$ mice, which were phenotypically normal. Some of these mice were bred with *Mox2-Cre* mice to delete exon 2 in the germ-line (*Gad1*$^{D/+}$). *Gad1*$^{D/+}$ mice were bred with each other, but no mice survived beyond the perinatal period, as was observed for with *Gad1*-null mice.

Supplementary figure 3



Supplementary Figure 3. Pyramidal cell somata innervated by a basket cell have similar perisomatic bouton densities independent of their locations in the innervation field and their distances from the basket cell soma

The relationship between perisomatic synapse density of a pyramidal cell soma and its distance from the basket cell soma that innervates it. For each experimental group, 7 basket cells were analyzed. Pyramidal cell somata were divided into 4 groups according to their distances from the basket cell soma (up to 300 μm). For each group, 4 to 8 pyramidal cell somata were analyzed for each basket cell. Data from basket cells in the same experimental group were pooled. Bars represent the average number of boutons per pyramidal cell soma plotted against its distance from the basket cell soma. Bouton density around pyramidal cell soma is independent of its distance from the basket cell soma (One-way Anova, *posthoc* Dunn's test, $p>0.05$).

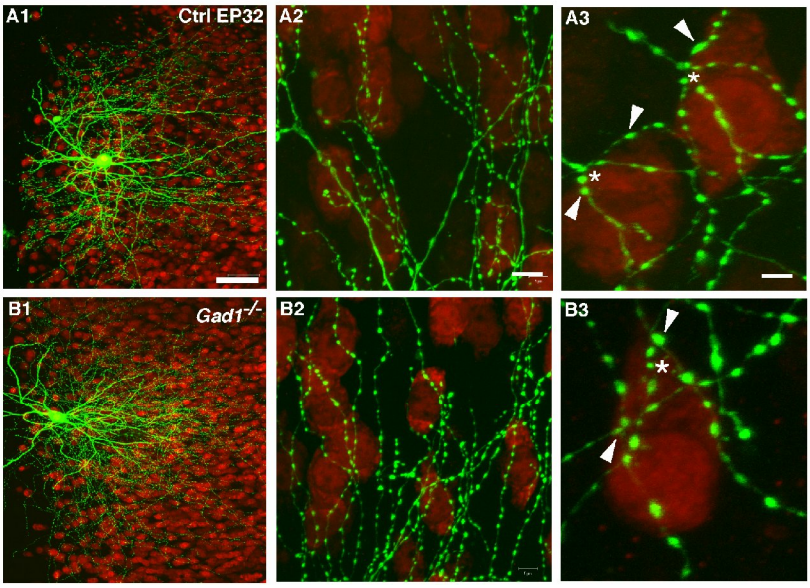


Supplementary Figure 4. GFP-positive boutons are presynaptic component of symmetric synapses.

A, Basket cells were co-transfected with P_{G67}-tdtomato (A1, red) and P_{G67}-syn-GFP (A2, green) to simultaneously visualize axon-bouton morphology and a synaptic marker. The vast majority of tdtomato-labeled boutons contained syn-GFP (A3, arrowheads). Scale bar: 5 μm.

B, More examples of Immuno-EM of typical GFP-labeled axonal boutons in control basket cells showing clear symmetric synaptic contacts (arrowheads), characterized by synaptic clefts, vesicle clustering, and thickening of the postsynaptic membrane, made on a dendritic shaft (**B1**) and soma (**B2**) of its postsynaptic target. Scale bar: 0.5μm.

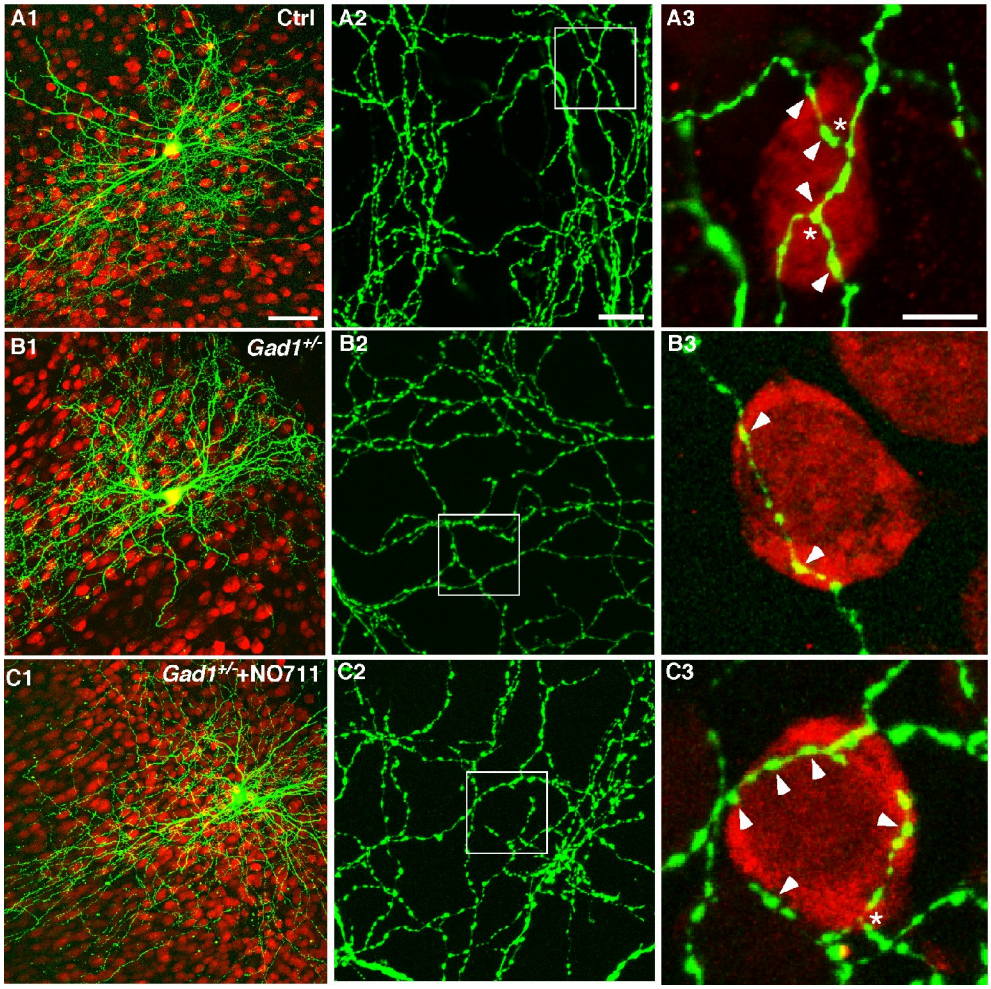
Supplementary figure 5



Supplementary Figure 5. Inactivation of *Gad1* from EP26-32 does not affect perisomatic synapse maintenance

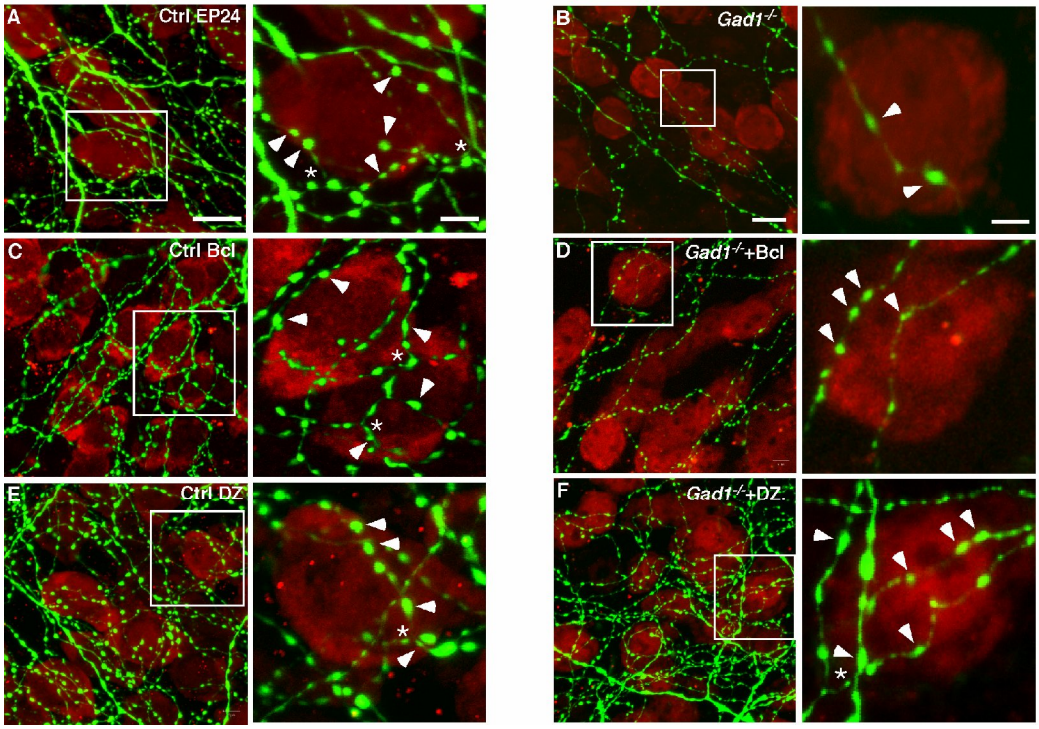
A-B, When *Gad1*$^{-/-}$ is deleted from EP26-32, axonal morphology of *Gad1*$^{-/-}$ cells (**B1**) is similar to aged-matched control cells (**A1**). Axon arbors of both *Gad1*$^{-/-}$ cells (**B1,2**) and control cells (**A1,2**) show extensive branching, dense boutons along axons (**A2, B2**), and terminal branches (asterisks, **A3, B3**) around pyramidal cell somata (NeuN immunostaining, red) with prominent and clustered boutons (arrowheads). Scale bar: **A1, B1**: 50 µm; **A2, B2**; 10 µm; **A3, B3**: 5 µm.

Supplementary figure 6



Supplementary Figure 6. Blocking of GABA transporter GAT1 rescues perisomatic innervation in *Gad*$^{+/-}$ basket cells. **A,** EP24 control basket cell. **B,** EP24 *Gad*$^{+/-}$ basket cell. **C**, Infusion of the GAT1 antagonist NO711 from EP16-24 rescues both bouton formation (arrowheads) and terminal branching (asterisks) in *Gad*$^{+/-}$ cells. Pyramidal somata are labeled by NeuN (red). A3, B3, C3 are from corresponding boxed region in A2, B2, C2. Scale bar: **A1, B1**: 50 µm; **A2, B2**; 10 µm; **A3, B3**: 5 µm.

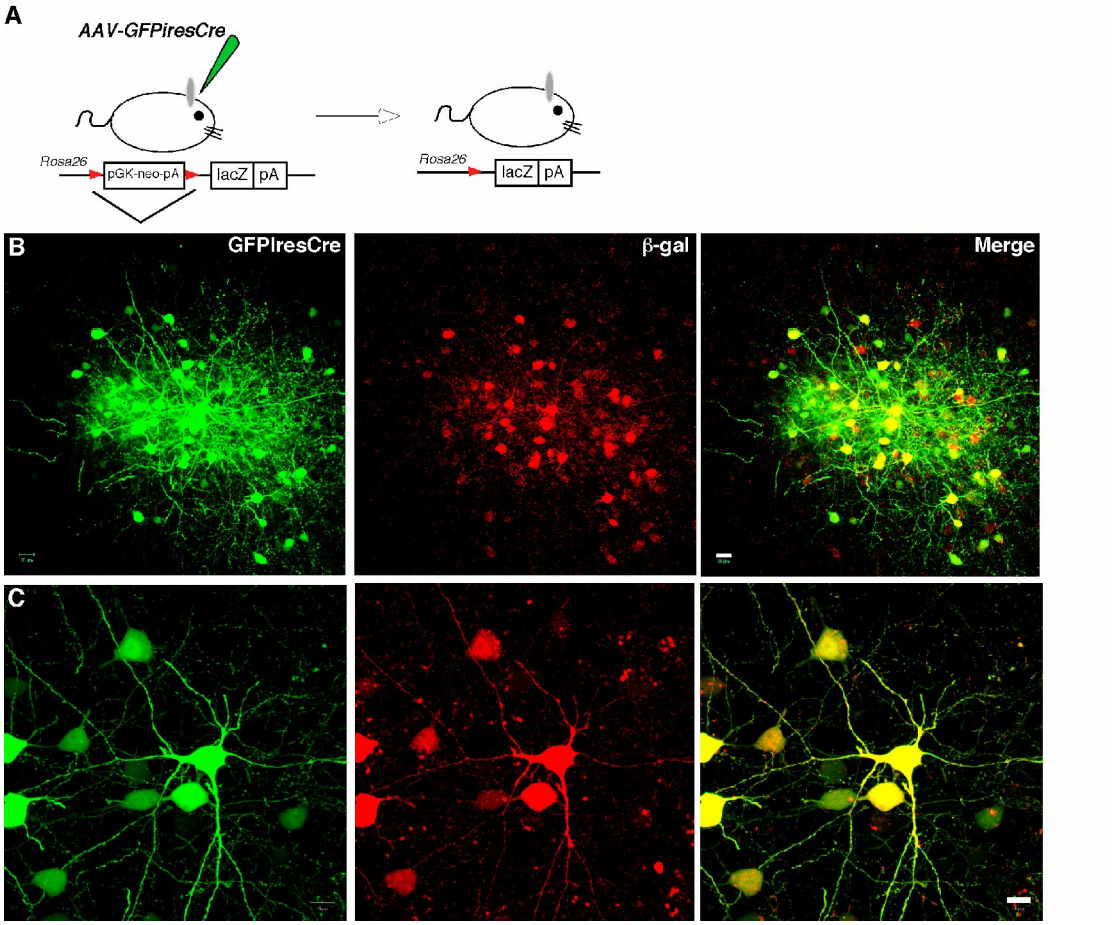
Supplementary figure 7



Supplementary Figure 7. Diazepam or Baclofen treatment from EP16-24 partially rescue different aspects of perisomatic innervation in *Gad1*$^{-/-}$ basket cells

A, EP24 control basket cell. **B,** EP24 *Gad1*$^{-/-}$ basket cell. **D**, Infusion of the $GABA_B$ agonist Baclofen (Bcl) partially rescues bouton formation in *Gad1*$^{-/-}$ cells (arrowheads), but not terminal branching (asterisks). **F**, Infusion of the $GABA_A$ agonist diazepam (DZ) rescues both terminal branching and bouton formation in *Gad1*$^{-/-}$ cells. Pyramidal somata are labeled by NeuN (red), **C, E**, Neither baclofen nor diazepam treatment affect perisomatic synapse maturation in control cells. Right panels are from corresponding boxed regions in left panels. Scale bar: left panels, 10 μm.; right panels, 5 μm.

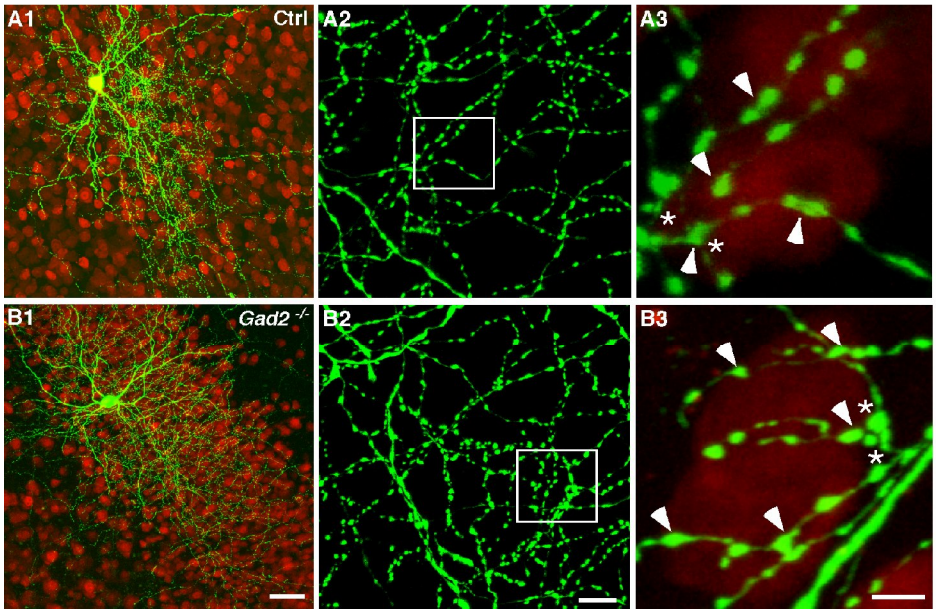
Supplementary figure 8



Supplementary Figure 8. ***AAV-GFP-ires-Cre*** **virus infection mediates recombination in neurons in visual cortex** ***in vivo.***

A, Visual cortex of floxed-*Rosa26-LacZ* reporter mouse mice (Soriano, 1999) was injected with *AAV-GFP-ires-Cre* at P15. Excision of the floxed cassette removes the stop codon and allows transcription of *LacZ*. **B-C,** Low (B) and high (C) magnification of the injection site, 18-days post injection. GFP expressing cells (green) show recombination induced ß-galactosidase expression (red). Scale bars: **B**, 20 µm; **C**, 10 µm.

Supplementary figure 9



Supplementary Figure 9. GAD65 is not required for maturation of perisomatic innervation

A, B, At EP27, basket interneurons transfected with P_{G67}-*GFP* in cortical organotypic cultures from germ-line GAD65-null (*Gad2*$^{-/-}$) mice are indistinguishable from those of wild-type (ctrl) littermates in their overall axon morphology (**A1-B1**), axon branching pattern (**A2-B2**) and perisomatic synapses (**A3-B3**). Arrowheads: boutons, asterisk: terminal branching. Scale bar: **A1**, **B1**: 50 µm; **A2**, **B2**: 10 µm; **A3**, **B3**: 5 µm