# Statistics reporting, by figure

- Please specify the following information for each panel reporting quantitative data, and where each item is reported (section, e.g. Results, paragraph number).
- Each figure legend should ideally contain an exact sample size (n) for each experimental group/condition, where n is an exact number and not a range, a clear definition of how n is defined (for example x cells from x slices from x animals from x litters, collected over x days), a description of the statistical test used, the results of the tests, any descriptive statistics and clearly defined error bars if applicable.
- For any experiments using custom statistics, please indicate the test used and stats obtained for each experiment.
- Each figure legend should include a statement of how many times the experiment shown was replicated in the lab; the details of sample collection should be sufficiently clear so that the replicability of the experiment is obvious to the reader.
- For experiments reported in the text but not in the figures, please use the paragraph number instead of the figure number.

Note: Mean and standard deviation are not appropriate on small samples, and plotting independent data points is usually more informative. When technical replicates are reported, error and significance measures reflect the experimental variability and not the variability of the biological process; it is misleading not to state this clearly.

<table>
<thead>
<tr>
<th>TEST USED</th>
<th>n</th>
<th>DESCRIPTIVE STATS</th>
<th>P VALUE</th>
<th>DEGREES OF FREEDOM &amp; F/T/Z/R/ETC VALUE</th>
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<tbody>
<tr>
<td>FIGURE</td>
<td>WHICH TEST</td>
<td>SECTION &amp; PARAGRAPH</td>
<td>VALUE</td>
<td>DEFINED</td>
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<tr>
<td>1a</td>
<td>one-way ANOVA</td>
<td>Fig. legend</td>
<td>9, 9, 10, 15</td>
<td>mice from at least 3 litters/group</td>
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<td>6</td>
<td>unpaired t-test</td>
<td>Results para 6</td>
<td>15</td>
<td>slices from 10 mice</td>
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<td>FIGURE NUMBER</td>
<td>WHICH TEST?</td>
<td>SECTION &amp; PARAGRAPH #</td>
<td>n</td>
<td>DESCRIPTIVE STATS (AVERAGE, VARIANCE)</td>
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<tr>
<td>Fig. 2c</td>
<td>Chi square test for homogeneity</td>
<td>Results 1st section 3rd para</td>
<td>68 and 228 dendritic cross-sections</td>
<td>Results 1st section 3rd para</td>
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<td>Fig. 2d</td>
<td>Linear regression</td>
<td>Results 1st section 3rd para</td>
<td>16 GlyT2+ boutons</td>
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<td>Fig. 3c</td>
<td>Wilcoxon signed rank test</td>
<td>Results 2nd section 1st para</td>
<td>7 slices from 4 mice</td>
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<td>Results 2nd section 4th para</td>
<td>7 slices from 3 mice</td>
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<td>Results 3rd section 1st para</td>
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<td>Fig. 5d</td>
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<td>Results 4th section 1st para</td>
<td>25 Stimulation section and preceding control periods</td>
<td>Fig. 5 legend</td>
</tr>
</tbody>
</table>
### Representative figures

1. Are any representative images shown (including Western blots and immunohistochemistry/staining) in the paper?
   
   If so, what figure(s)?

2. For each representative image, is there a clear statement of how many times this experiment was successfully repeated and a discussion of any limitations in repeatability?

   If so, where is this reported (section, paragraph #)?

<table>
<thead>
<tr>
<th>Figure</th>
<th>Mann-Whitney U-test</th>
<th>Suppl. fig. 8 legend</th>
<th>representative animal</th>
<th>Suppl. fig. 8 legend</th>
<th>mean +/- SEM</th>
<th>p</th>
<th>Suppl. fig. 8 legend</th>
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<td>165, 163</td>
<td>representative animal</td>
<td>Suppl. fig. 8 legend</td>
<td>mean +/- SEM</td>
<td>p&lt;0.00001</td>
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<td>mean +/- SEM</td>
<td>p&lt;0.00001</td>
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1. Figure 1, 2, 5 a-b, 6c
   Supplementary Fig. 1-3, 5a, b, 6, 8, 9, 10e

2. Figure 1 - Results, 1st section, 1-2nd para
   Figure 2 - Results, 1st section, 3-4th para
   Figure 3 - Results, 2nd section.
   Figure 4 - Results, 3rd section
   Figure 5a-b - Results, 4th section, 1st para
   Figure 6b-c - Results 4th section 2nd para
   Supplementary Fig. 1-3 - Results, 1st section, 1-2nd para
   Supplementary Fig. 5a - Results, 1st section, 4th para
   Supplementary Fig. 7 - Results, 2nd section, Suppl. Figure legend 7
   Supplementary Fig. 6 - Supplementary Figure legend 6
   Supplementary Fig. 8 - Supplementary Figure legend 8
   Supplementary Fig. 9 - Supplementary Figure legend 9
   Supplementary Fig. 10 - Results 4th section 2nd para
Statistics and general methods

1. Is there a justification of the sample size?
   If so, how was it justified?
   Where (section, paragraph #)?

Even if no sample size calculation was performed, authors should report why the sample size is adequate to measure their effect size.

In case of the anatomical experiments, representative number of cell bodies, axon terminals or receptor clusters were examined based on earlier data and our own earlier experience. Increasing the sample size did not change the ratio of thalamic projecting GlyT2-positive PnO cells or terminals. At the electron microscopic level co-localization with GABA was close to 100%. Quantitative measurements from 3D EM reconstructions of axon terminals revealed homogeneous features (Fig 1-2).

Concerning the in-vitro optogenetic experiments, more than 30 cells (n=32) in slices from 14 virus-injected animals were registered in different conditions and configurations to collect the data described in this study. In each series of experiments, the number of repetitions performed gave very solid and reproducible results. In the only case of a very variable outcome (the mixed GABA/glycinergic nature of the synaptic pathway under study), a very large cell sample (n=22) was collected to better describe the data and allow a meaningful comparison with our morphological correlates (Fig 3).

In case of in vivo optogenetic experiments the behavioural and EEG responses were robust with little variability (Fig 4-5).

In case of the in vivo recorded PnO cells other than phase coupling 8 out of the 11 cells showed homogeneous firing features. The variability of phase coupling is explicitly stated in the Results and shown in Fig 6e.

2. Are statistical tests justified as appropriate for every figure?
   Where (section, paragraph #)?

   a. If there is a section summarizing the statistical methods in the methods, is the statistical test for each experiment clearly defined?

   b. Do the data meet the assumptions of the specific statistical test you chose (e.g. normality for a parametric test)?
      Where is this described (section, paragraph #)?

   c. Is there any estimate of variance within each group of data?
      Is the variance similar between groups that are being statistically compared?
      Where is this described (section, paragraph #)?

All statistical tests were carefully selected based on mathematical reasons. In particular: Fig. 2c, anatomical target element distributions were compared using Chi square test for homogeneity as appropriate for discretized distributions. Fig. 3, IPSP comparisons were performed using the non-parametric Mann-Whitney U-test, as normality of the underlying distributions was not necessarily fulfilled. Fig. 4 and 5, Mann-Whitney U-test was used for comparisons of behavioral and EEG data. These tests are standard choices of the field, therefore we refrained from including a detailed justification.

The statistical tests applied are included in each corresponding method section separately.

Non-parametric statistical tests were mainly chosen for in-vitro and in-vivo optogenetic experiments with the clear purpose of not making assumptions of any kind on the distribution of the analyzed parameters. Since this is standard procedure in the field, we presently did not state this in the manuscript.

Variance was reported as standard deviation (target dendrite diameter, p. 6 para 2; number of receptor clusters, p. 7 para 1) or range (I(e)PSC amplitudes, p. 8 para 2). Accuracy of descriptive statistics was reported as standard error.
d. Are tests specified as one- or two-sided?

All tests were two sided as the statistical question was whether any difference was present (alternative hypothesis), with the exception of Fig. 5d, where the question whether optogenetic stimulation caused an increase in any spectral components required a one sided approach.

e. Are there adjustments for multiple comparisons?

The analyses performed in this study did not require the performance of multiple tests. The only exception was Fig. 5d, where spectra were compared at multiple frequencies. A lower p value threshold of p<0.01 was used to reduce the probability of false positives. The validity of the results were confirmed by the continuity of the frequency domain with significant increase and the overall low value of actual significant p values.

3. Are criteria for excluding data points reported?

Was this criterion established prior to data collection?

No data points were excluded.

Where is this described (section, paragraph #)?

4. Define the method of randomization used to assign subjects (or samples) to the experimental groups and to collect and process data.

If no randomization was used, state so.

Mice were randomly assigned to ChR2 and control groups. (This is standard procedure, therefore we did not specifically mention in the current version of the manuscript.)

Where does this appear (section, paragraph #)?

5. Is a statement of the extent to which investigator knew the group allocation during the experiment and in assessing outcome included?

If no blinding was done, state so.

Although blinding the experimenter for recording and stimulation experiments were not feasible, all data were subjected to automated data analysis treating recordings from different groups equally.

Where (section, paragraph #)?

6. For experiments in live vertebrates, is a statement of compliance with ethical guidelines/regulations included?

Yes, Materials and Methods - first para

Where (section, paragraph #)?

7. Is the species of the animals used reported?

Yes, Abstract

Where (section, paragraph #)?

8. Is the strain of the animals (including background strains of KO/ transgenic animals used) reported?

Yes, Extended Data, Materials and Methods, Generation of GlyT2-Cre BAC transgenic mice - first para

Where (section, paragraph #)?

9. Is the sex of the animals/subjects used reported?

Yes, Extended Data, Materials and Methods, - first para

Where (section, paragraph #)?

10. Is the age of the animals/subjects reported?

Yes, Extended Data, Materials and Methods, - first para

Where (section, paragraph #)?

Nature Neuroscience: doi:10.1038/nn.3951
11. For animals housed in a vivarium, is the light/dark cycle reported?
   Where (section, paragraph #)?
   No

12. For animals housed in a vivarium, is the housing group (i.e. number of animals per cage) reported?
   Where (section, paragraph #)?
   No

13. For behavioral experiments, is the time of day reported (e.g. light or dark cycle)?
   Where (section, paragraph #)?
   No

14. Is the previous history of the animals/subjects (e.g. prior drug administration, surgery, behavioral testing) reported?
   Where (section, paragraph #)?
   Yes, Extended Data, Materials and Methods, In vivo physiology, first para

   a. If multiple behavioral tests were conducted in the same group of animals, is this reported?
      Where (section, paragraph #)?
      N/A

15. If any animals/subjects were excluded from analysis, is this reported?
   Where (section, paragraph #)?
   No animals were excluded.

   a. How were the criteria for exclusion defined?
      Where is this described (section, paragraph #)?

   b. Specify reasons for any discrepancy between the number of animals at the beginning and end of the study.
      Where is this described (section, paragraph #)?

Reagents

1. Have antibodies been validated for use in the system under study (assay and species)?
   Yes

   a. Is antibody catalog number given?
      Where does this appear (section, paragraph #)?

   The following antibodies have been used in the manuscript:
   (name/company/Cat. No.)
   1. rabbit anti-fluorogold - Chemicon - AB 153
   2. mouse anti-eGFP - Invitrogen (Molecular Probes) - A11120
   3. guinea pig anti-GlyT2 - Chemicon AB 1773
   4. rabbit anti-GABA-A receptor gamma2 - Synaptic Systems, 224 003
   5. mouse anti-Glycine receptor alpha 1- Synaptic Systems, 146 011
   6. rabbit anti-GABA - gift of Prof P. Somogyi - Oxford

   Companies and species in which the antibodies were raised are reported in Materials and Methods, Immunocytochemistry section. These data clearly identify the antibodies.
b. Where were the validation data reported (citation, supplementary information, Antibodypedia)?

Where does this appear (section, paragraph #)?

In case of antibody 1 and 2 the specificity was described by the companies of origin. Fluorogold and eGFP are normally not present in the brain. Fluorogold signal was found only in the injections sites and in the projecting cell bodies, nowhere else in the brain. The eGFP antibodies labeled only eGFP-positive structures in double fluorescent stainings of GlyT2::eGFP animals.

In case of anti-GlyT2 (antibody 3) beside the specificity tests provided by the company, close to 100% co-localization with cell bodies and axons of GlyT2-positive elements in two GlyT2 mouse lines (Glyt2::eGFP and GlyT2-Cre, Extended Data, Materials and Methods, Immunocytochemistry 3rd para and Generation of GlyT2-Cre BAC transgenic line, Extended Data Fig 6.) confirmed the specificity of the antibody.

In case of antibody 4 we relied on the specificity tests of the company. In addition, we performed tests using focal, viral mediated, conditional knock out of the gamma2 gene. Lack of staining in the virus infected regions demonstrated the specificity of the antibody (reported in Rovó et al., 2014 JNsci, 34:7137).

In case of antibody 5 we relied on the specificity tests of the company and earlier studies. The distribution of the immunolabeling was identical with the mRNA signal of GlyR1. KO tests are presently not available due to the lethality of the mouse line. Extensive, punctate co-localization of GlyR with GABA-A gamma2 subunit postsynaptic to GlyT2 terminals (Fig 2D and Extended Data Fig5, quantified using a custom made algorithm, Fig2E) strongly suggest the specificity of this antibody, which is the major antibody used in the literature (known as mAb4a after the clone).

Finally the 6th antibody was originally characterized by Somogyi et al (1985) as cited in Extended data, Materials and Methods, Electron microscopy. Further data on the specificity is available in Giber et al. JCN, 2008, 506:122).

2. If cell lines were used to reflect the properties of a particular tissue or disease state, is their source identified?

Where (section, paragraph #)?

N/A

a. Were they recently authenticated?

Where is this information reported (section, paragraph #)?

N/A

Data deposition

Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Microarray data

Deposition is strongly recommended for many other datasets for which structured public repositories exist; more details on our data policy are available here. We encourage the provision of other source data in supplementary information or in unstructured repositories such as Figshare and Dryad.

1. Are accession codes for deposit dates provided?

Where (section, paragraph #)?

N/A
Computer code/software

Any custom algorithm/software that is central to the methods must be supplied by the authors in a usable and readable form for readers at the time of publication. However, referees may ask for this information at any time during the review process.

1. Identify all custom software or scripts that were required to conduct the study and where in the procedures each was used.

The analysis of the confocal images following immunohistochemical procedures (Fig. 2e) was performed using both custom scripts (to isolate varicosities and iteratively inflate them to measure distances to receptor clusters), written in imageJ macros and using the following plugins: Object Counter 3D, 3D Roi manager, Iterative Deconvolve 3D. R and matlab were used for further statistical analysis, and for representing these data graphically respectively. Quantitative analysis of the in-vitro, and in-vivo optogenetic experiments was performed using custom-developed routines written in Igor (Wavemetrics, Lake Oswego, USA).

2. Is computer source code/software provided with the paper or deposited in a public repository? Indicate in what form this is provided or how it can be obtained.

All the source codes for custom-developed software are readily obtainable upon request to the corresponding authors.

Human subjects

1. Which IRB approved the protocol?
   Where is this stated (section, paragraph #)?

Regional and Institutional Committee of Science and Research Ethics of Scientific Council of Health (ETT TUKEB 31443/2011/EKU (518/PI/11)
Materials and Methods, Processing of Human Tissues - first para

2. Is demographic information on all subjects provided?
   Where (section, paragraph #)?

Yes, Materials and Methods, Processing of Human Tissues - first para

3. Is the number of human subjects, their age and sex clearly defined?
   Where (section, paragraph #)?

Yes, Results, First section and Materials and Methods, Processing of Human Tissues - first para

4. Are the inclusion and exclusion criteria (if any) clearly specified?
   Where (section, paragraph #)?

Patients who died from causes linked to brain diseases or had a history of any neurological disorders were not included. Materials and Methods, Processing of Human Tissues - first para

5. How well were the groups matched?
   Where is this information described (section, paragraph #)?

N/A

6. Is a statement included confirming that informed consent was obtained from all subjects?
   Where (section, paragraph #)?

Yes, Materials and Methods, Processing of Human Tissues - first para
7. For publication of patient photos, is a statement included confirming that consent to publish was obtained?  
Where (section, paragraph #)?  
N/A

fMRI studies

For papers reporting functional imaging (fMRI) results please ensure that these minimal reporting guidelines are met and that all this information is clearly provided in the methods:

1. Were any subjects scanned but then rejected for the analysis after the data was collected?  
   a. If yes, is the number rejected and reasons for rejection described?  
      Where (section, paragraph #)?

2. Is the number of blocks, trials or experimental units per session and/or subjects specified?  
   Where (section, paragraph #)?

3. Is the length of each trial and interval between trials specified?

4. Is a blocked, event-related, or mixed design being used? If applicable, please specify the block length or how the event-related or mixed design was optimized.

5. Is the task design clearly described?  
   Where (section, paragraph #)?

6. How was behavioral performance measured?

7. Is an ANOVA or factorial design being used?

8. For data acquisition, is a whole brain scan used?  
   If not, state area of acquisition.  
   a. How was this region determined?

9. Is the field strength (in Tesla) of the MRI system stated?  
   a. Is the pulse sequence type (gradient/spin echo, EPI/spiral) stated?  
   b. Are the field-of-view, matrix size, slice thickness, and TE/TR/flip angle clearly stated?
10. Are the software and specific parameters (model/functions, smoothing kernel size if applicable, etc.) used for data processing and pre-processing clearly stated?

11. Is the coordinate space for the anatomical/functional imaging data clearly defined as subject/native space or standardized stereotaxic space, e.g., original Talairach, MNI305, ICBM152, etc? Where (section, paragraph #)?

12. If there was data normalization/standardization to a specific space template, are the type of transformation (linear vs. nonlinear) used and image types being transformed clearly described? Where (section, paragraph #)?

13. How were anatomical locations determined, e.g., via an automated labeling algorithm (AAL), standardized coordinate database (Talairach daemon), probabilistic atlases, etc?

14. Were any additional regressors (behavioral covariates, motion etc) used?

15. Is the contrast construction clearly defined?

16. Is a mixed/random effects or fixed inference used?
   a. If fixed effects inference used, is this justified?

17. Were repeated measures used (multiple measurements per subject)?
   a. If so, are the method to account for within subject correlation and the assumptions made about variance clearly stated?

18. If the threshold used for inference and visualization in figures varies, is this clearly stated?

19. Are statistical inferences corrected for multiple comparisons?
   a. If not, is this labeled as uncorrected?

20. Are the results based on an ROI (region of interest) analysis?
   a. If so, is the rationale clearly described?
   b. How were the ROI’s defined (functional vs anatomical localization)?

21. Is there correction for multiple comparisons within each voxel?
22. For cluster-wise significance, is the cluster-defining threshold and the corrected significance level defined?

Additional comments

Additional Comments