Supplemental Information

Differentiating Cerebellar Impact on Thalamic Nuclei

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Differentiating cerebellar impact on thalamic nuclei

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Supplementary information

Experimental procedures                      page 1
Table S1 – related to Fig. 1                  page 8
Table S2 – related to Fig. 2                  page 9
Table S3 – related to Fig. 3                  page 10
Table S4 – related to Fig. 4                  page 12
Table S5 – related to Fig. 5                  page 13
Table S6 – related to Fig. 6                  page 14
Table S7 – related to Fig. 7                  page 15
Experimental Procedures

All experiments were performed in accordance with the European Communities Council Directive. Protocols were reviewed and approved by the Dutch national experimental animal committees (DEC) and every precaution was taken to minimize stress, discomfort and the number of animals used.

Animals

Data were collected from 21-56 day old C57BL/6NHsd mice of both sexes, which were purchased from Envigo laboratories (Horst, Netherlands).

Viral injections

Mice were anesthetized with isoflurane, (4% in 0.5 L/min O2 for induction and 1.5% in 0.5 L/min O2 for maintenance), carprofen (5 mg/kg), buprenorphine (50 µg/kg) and lidocaine (10%, local application). For optogenetic stimulation we stereotactically delivered adeno-associated virus (AAV) encoding Channelrhodopsin2 (ChR2) coupled with a EYFP fluorophore (AAV2-hSyn-ChR2(H134R)-EYFP) to the CN. Following bilateral craniotomies of ~0.5 mm above the interparietal bone (-2 mm anterior-posterior and 1.5-2 mm medial-lateral to lambda), 150-200 nl (at a rate of ~20 nl/min) of AAV was injected to the CN in both hemispheres. The viral vector was kindly provided by Prof. K. Deisseroth (Stanford University) through the UNC and UPENN vector cores.

Preparation of acute brain slices

Following 4-6 weeks of incubation isoflurane-anesthetized mice were decapitated, their brains were quickly removed and placed into ice-cold slicing medium containing (in mM): 93 NMDG, 93 HCl, 2.5 KCl, 1.2 NaHPO4, 30 NaHCO3, 25 Glucose, 20 HEPES, 5 Na-ascorbate, 3 Na-pyruvate, 2 Thiourea, 10 MgSO4, 0.5 CaCl2, 5 N-acetyl-L-Cysteine (osmolarity 310 ± 5; bubbled with 95% O2 / 5% CO2)
(Ting et al., 2014). Next, 250-300 μm thick horizontal or coronal slices were cut using a Leica vibratome (VT1000S). For the recovery, brain slices were incubated for 5 min in slicing medium at 34 ± 1°C and subsequently for ~40 min in aCSF (containing in mM: 124 NaCl, 2.5 KCl, 1.25 Na2HPO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, and 20 D-glucose, osmolarity 310 ± 5; bubbled with 95% O2 / 5% CO2) at 34 ± 1°C. After recovery brain slices were stored at room temperature (RT) before the experiments started. The accompanying hindbrain was post-fixed in 4% paraformaldehyde (PFA), for histological confirmation of the viral injection location (see below).

**In vitro whole cell recordings**

For all recordings, slices were bathed in 34 ± 1°C ACSF (bubbled with 95% O2 and 5% CO2). Whole-cell patch-clamp recordings were performed using an EPC-9 or EPC-10 amplifier (HEKA Electronics, Lambrecht, Germany) for 20-60 min and digitized at 20 kHz. Resting membrane potential (Vrest) and input resistance (Rin) were recorded after whole-cell configuration was reached. Recordings were excluded if the series resistance (Rs) (assessed by -5 or -10 mV voltage steps following each test pulse) varied by >25% over the course of the experiment. Voltage and current clamp recordings were performed using borosilicate glass pipettes with a resistance of 3-5 MΩ when filled with K+-based internal (in mM: 124 K-Gluconate, 9 KCl, 10 KOH, 4 NaCl, 10 HEPES, 28.5 Sucrose, 4 Na2ATP, 0.4 Na3GTP (pH 7.25-7.35; osmolarity ~290)). Recording pipettes were supplemented with 1 mg/ml biocytin to allow histological staining (see below). Current clamp recordings were corrected offline for the calculated liquid junction potential of -10.2 mV. All recordings were performed in the presence of picrotoxin (100 μM, Sigma-Aldrich) to block GABAA-receptor-mediated IPSCs.

Full-field optogenetic stimulation (470 nm peak excitation) was generated using a Polygon4000 (Mightex, Toronto, Canada) or a pE2 (CoolLED, Andover, UK), that were controlled using TTL-pulses generated by the HEKA amplifier. Light intensities at 470 nm were recorded using a photometer (Newport 1830-C equipped with an 818-ST probe, Irvine, CA) at the level of the slice. Typically the light intensities sufficient to trigger the maximal response amplitude in thalamic cells ranged from 0.1 to 6.65 mW/mm². To trigger neurotransmitter release from transfected CN axons we delivered 1 ms light pulses at 0.1 Hz and an intensity resulting in the maximally evoked response,
unless stated otherwise. To characterize the postsynaptic receptors we sequentially bath-applied AMPA- (10 μM NBQX), NMDA- (10 μM APV), mGluR1- (10 μM JNJ-16259685) and mGluR5- (50 μM MPEP) blockers. Each drug was added only after the EPSC amplitude stabilized. All drugs were purchased from Tocris (Bristol, UK). To ensure that we recorded action potential-driven neurotransmitter release most experiments were concluded by bath application of 10 μM tetrodotoxin (TTX), which blocked all post-synaptic responses in the recorded thalamic neurons. The responses evoked in thalamic neurons by optogenetic stimulation of CN axons were solely of monosynaptic origin, which matches the known absence of local interneurons and of local axon collaterals in the nuclei we studied (Jones, 2007).

**Immunofluorescence**

To visualize the recorded neurons and CN axons, slices were placed in 4% PFA (in 0.12 M PB) for at least 24 hrs (Marx et al., 2012). Subsequently, slices were transferred into 0.1 M PBS and rinsed with PBS 3 times for 10 min. Slices were incubated for 1 hr at RT in blocking solution (containing 10% normal horse serum (NHS) and 0.5% triton diluted in PBS), which was followed by over-night incubation with primary antibody for vesicular glutamate transporter type 2 (vGluT2) (anti Guinea Pig; Millipore Bioscience Research Reagent; 1:2000 diluted in PBS containing 2% NHS and 0.4% Triton). Slices were subsequently rinsed 3 times for 10 min and incubated for 2 hrs with Streptavidin-Cy3 (1:200, Jackson Immunoresearch) and anti Guinea Pig Cy5 (1:200, Jackson Immunoresearch) diluted in PBS containing 2% normal horse serum and 0.4% triton. Sections were rinsed in PBS, mounted with Vectashield (Vector laboratories) and imaged with a LSM 700 confocal microscope (Carl Zeiss Microscopy, LLC, USA).

For localization of the injection sites, the cerebellum was removed from forebrain and fixed with PFA 4% for 5 to 10 days on a shaker at 4°C. Serial 40 μm thick horizontal sections were obtained on a freezing microtome. The tissue was rinsed in PBS solution and then transferred in blocking solution for 1 hr at RT and subsequently incubated for 10 min with DAPI (300nM). Sections where rinsed with 0.01M PB and mounted on glass.
Fluorescence microscopy and reconstruction

Guided by calbindin D28-K staining (primary: Calbindin α-rabbit 1:7000, Swant Inc, #CB-38a; secondary: 405 nm rabbit-α-donkey 1:400, Jackson Immunoresearch #A421) and a reference atlas (Franklin and Paxinos, 2001) we outlined the thalamic nuclei of interest. For each nucleus the expression pattern of ChR2-YFP was quantified with RGB measure function of Fiji (ImageJ) in order to have the mean intensity among the region of interest (ROI).

Recorded neurons were labeled with biocytin. Epifluorescent tile images were obtained using a 20X/0.30 NA (air) objective and a LSM 700 microscope (Carl Zeiss). The position of labeled neurons was confirmed using a stereotactic atlas (Franklin and Paxinos, 2001). Terminals positive to VGluT2 staining were identified and morphologically studied using confocal images that were captured using the following excitation wavelengths: 488 nm (YFP), 555 nm (Cy3) and 639 nm (Cy5). Terminals were imaged using a 40X/1.30 NA (oil) objective by acquiring a stack of images with 0.5 digital zoom and a voxel size of 313 nm width x 313 nm length x 300 nm depth. Using custom-written Fiji-scripts (ImageJ) we identified putative synaptic contacts, i.e. YFP-positive varicosities that colocalized with vGluT2-staining that are within 1 µm distance from the recorded neurons. Once synaptic contacts were isolated high resolution image stacks were acquired using a 63X1.4 NA oil objective with 1X digital zoom, a pinhole of 1 Airy unit and significant oversampling for deconvolution (voxel dimension is: 46 nm width x 46 nm length x 130 nm depth calculated according to Nyquist factor; 8-bit per channel; image plane 2048 x 2048 pixels). Signal-to-noise ratio was improved by 2 times line averaging. Stack’s subsets of the connection were deconvolved using Huygens software (Scientific Volume Imaging). Further analysis was performed using a custom-written Fiji macro. The color channels (YFP, Cy3 and Cy5) of the images were split to get separate stacks. The YFP and Cy3 channels were Gaussian blurred (sigma = 1) and selected by a manually set threshold. A binary open function was done on both images (iterations = 4, count = 2) and objects were removed if their size was <400 pixels (YFP) or <120 pixels (Cy3). A small dilatation was done on the red image (iteration = 1, count = 1). With the image calculator an ‘and-operation’ was done
using the binary red and green image. The values 255 (white) of the binary YFP image were set to 127. This image and the result of the AND-operation were combined by an OR-operation. The resulting image was measured with the 3D-object counter plugin for volumes and maximum intensities. Only objects containing pixels with an intensity of 255 (overlap) are taken in account for analysis. Estimation of synapse density (number of terminals/area µm²) was obtained for each nucleus by dividing the number of terminals by the image area (DeKosky and Scheff, 1990). To quantify the distance from soma for vGluT2-positive CN terminals on reconstructed neurons we used a custom-written macro in Fiji software (ImageJ). Briefly, we calculated the distance in 3 dimensions (using x-, y-, z-coordinates) between the center of the terminal and the center of the soma by Pythagorean Theorem.

**3D Sholl analysis**

To determine the dendritic arborization of biocytin filled cells, we used the 3D Sholl analysis macro implemented in Fiji software (Ferreira et al., 2014). For preprocessing, image stacks over a z-volume of 18.5 - 87.5 µm were binarized. Stacks with excessive background signal were excluded from further analysis. Subsequently the dendritic arborization was measured in concentric shells of 10 µm distance starting with 15 µm distance from the center of the soma. At this first sphere we manually counted the number of primary dendrites and assessed their directionality by calculating the radial angle between the primary dendrites.

**Electron microscopy**

Four mice were injected with anterograde neuronal tract tracer biotinylated dextran–amine (10% BDA in 0.1 M PB, pH 7.4, molecular weight 10,000) by iontophoresis (pulses of 4 µA, 10 min) with a glass micropipette (tip opening, 8–10 µm) in the interpositus and lateral CN. After 5 days mice were anesthetized with an overdose of nembutal (i.p.) and transcardially perfused with 4% PFA and 1% glutaraldehyde in phosphate buffer. Brains were removed, kept overnight in 4% PFA, and cut into 60
μm thick coronal sections using a vibratome. Sections were subsequently washed in PBS, incubated for 20 min in 3% H₂O₂ (in PBS) to remove endogenous peroxidase activity of blood, washed again, placed for 1 hr in 10% NHS and finally incubated for 24 hrs in ABC-HRP (Vector). At the end of the immunostaining, the sections were stained with 0.5% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂ for 15 min at RT. Ultimately, the sections were osmicated with 1% osmium in 8% glucose solution, dehydrated in propylene oxide, and embedded in araldite (Durcupan, Fluka, Germany). Guided by staining levels in semithin sections (0.5 μm thick), we made pyramids of the VL, VM and CL nuclei. Ultrathin sections (60 nm) were cut using an ultramicrotome (Leica, Germany), mounted on nickel grids, and counterstained with uranyl acetate and lead citrate. CN axon terminals were photographed at various magnifications (range 3900X-25500X) using an electron microscope (CM 100, Philips, Eindhoven, Netherlands) and analyzed off-line using standard measurement functions in Fiji (ImageJ). To limit the possibility that our electron micrographs contained various images of the same pre- and postsynaptic structures we separated our ultrathin sections by various semi-thin sections.

Data analysis and statistics

Current and potential traces were acquired using Pulse and Patchmaster software (HEKA) and stored for offline analysis. Single stimulus data was analyzed using Clampfit software (Molecular Devices), while trains of stimuli were analyzed with custom written routines in Igor Pro 6.1 (Wavemetrics, Lake Oswego, Oregon). To evaluate the variability of EPSC amplitude and charge transfer we calculated the coefficient of variation (CV): the ratio between standard deviation and mean. For trains of stimuli, the peak amplitude of each evoked postsynaptic current (EPSC) was detected relative to baseline. All EPSC amplitudes within the train were normalized to the first EPSC. The total charge during train stimulation was calculated by determining the area under the curve between the first and the last stimulus relative to baseline. For all recordings averages of at least 5 sweeps per cell were calculated. The steady state amplitude was calculated by averaging the amplitude of responses to the last 5 stimuli.
Using GraphPad PRISM and SPSS software packages we ran statistical comparisons between the thalamic nuclei (VL, VM and CL) by one-way ANOVA, Kruskal-Wallis (K-W) or Kolmogorov-Smirnov (K-S) tests as indicated in the main text. Statistical difference for pharmacology data was assessed using Friedman test. For Sholl analysis a two-way ANOVA was used with Mann Whitney multiple comparison test. We corrected missing values by the Last observation carried forward (LOCF) method. Correlation coefficients were calculated using Spearman. We defined \( p<0.05 \) as a significant difference. Throughout the main text we report a subset of the statistical data; all details are provided in the Supplemental data tables that accompany each figure. Summarized data are represented as mean ± standard error of the mean. Throughout the figures data from VL are indicated in green, VM in red and CL in blue, unless stated otherwise.

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| Panel | Test applied | P-value | Degrees of freedom | Population size | Definition of population | Correction |
|-------|--------------|---------|--------------------|-----------------|--------------------------|------------|
| 1B    | Kruskal Wallis | 0.003   | 2                  | 18              | 6 mice                  | Dunn-Sidak |
|       |               |         | -VL-VM 0.529       |                 |                          |            |
|       |               |         | -VM-CL 0.136       |                 |                          |            |
|       |               |         | -VL-CL 0.002       |                 |                          |            |
| 1I    | Kolmogorov Smirnov | VL-VM <0.001 | 671 terminals    | 5 mice          | Bonferroni               |            |
|       |               |         | VM-CL 0.966        | 245 terminals   |                          |            |
|       |               |         | VL-CL <0.001       | 572 terminals   |                          |            |
| 1J    | Kruskal Wallis | 0.028   | 2                  | 15              | 5 mice                  | Dunn-Sidak |
|       |               |         | VL-VM 0.334        |                 |                          |            |
|       |               |         | VM-CL 0.865        |                 |                          |            |
|       |               |         | VL-CL 0.024        |                 |                          |            |
| Panel | Test applied | P-value | Degrees of freedom | Population size | Definition of population | Correction |
|-------|--------------|---------|--------------------|-----------------|--------------------------|------------|
| 2B EPSC Amplitude | Kruskal Wallis | $<0.001$ | 2 | 44 cells | 40 mice | Dunn-Sidak |
| | | &nbsp;&nbsp;&nbsp;&nbsp;VL-VM $0.001$ &nbsp;&nbsp;&nbsp;&nbsp;VM-CL 1 &nbsp;&nbsp;&nbsp;&nbsp;VL-CL $<0.001$ | | | | |
| 2B EPSC Charge | Kruskal Wallis | $<0.001$ | 2 | 47 cells | 40 mice | Dunn-Sidak |
| | | &nbsp;&nbsp;&nbsp;&nbsp;VL-VM $0.002$ &nbsp;&nbsp;&nbsp;&nbsp;VM-CL 1 &nbsp;&nbsp;&nbsp;&nbsp;VL-CL $0.001$ | | | | |
| 2D EPSC CV | Kruskal Wallis | $0.001$ | 2 | 42 cells | 40 mice | Dunn-Sidak |
| | | &nbsp;&nbsp;&nbsp;&nbsp;VL-VM $0.031$ &nbsp;&nbsp;&nbsp;&nbsp;VM-CL 1 &nbsp;&nbsp;&nbsp;&nbsp;VL-CL $0.001$ | | | | |
| 2D Charge CV | Kruskal Wallis | $0.001$ | 2 | 46 cells | 40 mice | Dunn-Sidak |
| | | &nbsp;&nbsp;&nbsp;&nbsp;VL-VM $0.025$ &nbsp;&nbsp;&nbsp;&nbsp;VM-CL 1 &nbsp;&nbsp;&nbsp;&nbsp;VL-CL $0.03$ | | | | |
### Table S3 – Statistical analysis of all data related to Figure 3

| Panel | Test applied | P-value | Degrees of freedom | Population size | Definition of population | Correction |
|-------|--------------|---------|--------------------|-----------------|----------------------------|------------|
| 3H    | **VL**       | Kruskal Wallis | **0.002**       | 2               | 39 cells                  | 29 mice    | Dunn-Sidak |
|       |              |         | 10-20 Hz 0.492    |                 |                            |            |
|       |              |         | 10-50 Hz **0.001**|                 |                            |            |
|       |              |         | 20-50 Hz 0.111    |                 |                            |            |
|       | **VM**       | Kruskal Wallis | 0.496           | 2               | 22 cells                  | 9 mice     |            |
|       |              |         |                   |                 |                            |            |
|       | **CL**       | Kruskal Wallis | **0.006**       | 2               | 16 cells                  | 9 mice     | Dunn-Sidak |
|       |              |         | 10-20 Hz 0.234    |                 |                            |            |
|       |              |         | 10-50 Hz **0.004**|                 |                            |            |
|       |              |         | 20-50 Hz 0.230    |                 |                            |            |
| 3H    | **10 Hz**    | Kruskal Wallis | 0.344           | 2               | 29 cells                  | 18 mice    |            |
|       |              |         |                   |                 |                            |            |
|       | **20 Hz**    | Kruskal Wallis | 0.168           | 2               | 27 cells                  | 13 mice    |            |
|       |              |         |                   |                 |                            |            |
|       | **50 Hz**    | Kruskal Wallis | 0.137           | 2               | 20 cells                  | 16 mice    |            |
| 3I    | **VL**       | Kruskal Wallis | 0.007           | 2               | 29 cells                  | 29 mice    | Dunn-Sidak |
|       |              |         | 10-20 Hz 0.464    |                 |                            |            |
|       |              |         | 10-50 Hz **0.005**|                 |                            |            |
|       |              |         | 20-50 Hz 0.529    |                 |                            |            |
|       | **VM**       | Kruskal Wallis | **0.037**       | 2               | 12 cells                  | 9 mice     | Dunn-Sidak |
|       |              |         | 10-20 Hz 1        |                 |                            |            |
|       |              |         | 10-50 Hz 0.056    |                 |                            |            |
|       |              |         | 20-50 Hz 0.118    |                 |                            |            |
|       | **CL**       | Kruskal Wallis | 0.077           | 2               | 12 cells                  | 9 mice     |            |
|       |              |         |                   |                 |                            |            |
| 3I    | **10 Hz**    | Kruskal Wallis | 0.167           | 2               | 20 cells                  | 18 mice    |            |
|       |              |         |                   |                 |                            |            |
|       | **20 Hz**    | Kruskal Wallis | 0.321           | 2               | 18 cells                  | 13 mice    |            |
| Hz  | Test  | Value | Group 1 | Group 2 |
|-----|-------|-------|---------|---------|
| 50  | Wallis| 0.867 | 2       | 15 cells| 16 mice |
Table S4 – Statistical analysis of all data related to Figure 4

| Panel 4B | Test applied | P-value | Degrees of freedom | Population size | Definition of population | Correction |
|----------|--------------|---------|--------------------|-----------------|--------------------------|------------|
| Control vs NBQX | Friedman | 0.500 | 3 | 10 cells | 9 mice | * |
| NBQX vs NBQX+APV | Friedman | 0.146 | 3 | 10 cells | 9 mice | Dunn-Bonferroni |
| Control vs NBQX+APV | Friedman | <0.001 | 3 | 10 cells | 9 mice | Dunn-Bonferroni |
| NBQX+APV vs NBQX-AP5-MPEP+JNJ | Friedman | 1.000 | 3 | 10 cells | 9 mice | Dunn-Bonferroni |
| NBQX vs NBQX+APV+MPEP+JNJ | Friedman | 0.019 | 3 | 10 cells | 9 mice | Dunn-Bonferroni |

* [http://www-01.ibm.com/support/docview.wss?uid=swg21508972](http://www-01.ibm.com/support/docview.wss?uid=swg21508972)
| Panel          | Test applied       | P-value       | Degrees of freedom | Population size | Definition of population | Correction    |
|---------------|--------------------|---------------|--------------------|-----------------|--------------------------|---------------|
| 5D            | 2-way ANOVA        | VL-VM 0.733   | 2                  | 26 cells        | 26 mice                  |               |
| Intersection  |                    | VM-CL 0.350  |                    |                 |                          |               |
| 55 µm         |                    | VL-CL 0.018   |                    |                 |                          |               |
| 5E            | Kruskal Wallis     | 0.015         | 2                  | 33 cells        | 26 mice                  | Dunn-Sidak   |
| 5F            | Kruskal Wallis     | 0.141         | 2                  | 164             | 26 mice                  |               |
| 5G            | Kolmogorov-Smirnov | VL-VM 0.831   | 93                 | 26 mice         | Bonferroni               |               |
|               |                    | VM-CL 0.136   | 123                |                 |                          |               |
|               |                    | VL-CL 0.343   | 112                |                 |                          |               |
Table S6 – Statistical analysis of all data related to Figure 6

| Panel | Test applied   | P-value | Degrees of freedom | Population size | Definition of population | Correction |
|-------|----------------|---------|--------------------|-----------------|--------------------------|------------|
| 6B    | Kruskal Wallis | 0.373   | 2                  | 34 cells        | 24 mice                  | Dunn-Sidak |
| 6C    | Kruskal Wallis | 0.586   | 2                  | 110 cells       | 24 mice                  | Dunn-Sidak |
| 6E    | Kruskal Wallis | **0.027** | 2                  | 130 terminals   | 24 mice                  | Dunn-Sidak |
|       |                |         |                    |                 |                          |            |
|       |                |         |                    |                 |                          |            |
| 6F    | Kolmogorov Smirnov | VL-VM 0.834 | 93 terminals      | 24 mice        | Bonferroni               |            |
|       |                | VM-CL 0.080 | 59 terminals      |                 |                          |            |
|       |                | **VL-CL 0.044** | 108 terminals    |                 |                          |            |
Table S7 – Statistical analysis of all data related to Figure 7

| Panel 7B | Test applied      | P-value | Degrees of freedom | Population size | Definition of population | Correction |
|----------|-------------------|---------|--------------------|-----------------|--------------------------|------------|
| Terminal surface | Kruskal Wallis | 0.099   | 2                  | 97 terminals    | 4 mice                  | Dunn Sidak |
| Number of Mitochondria | Kruskal Wallis | 0.468   | 2                  | 83 terminals    | 4 mice                  | Dunn Sidak |
| Mitochondria Surface | Kruskal Wallis | <0.001  | 2                  | 315 mitochondria | 4 mice                | Dunn Sidak |
|          |                   |         |                    | 315 mitochondria |                        |            |
|          |                   |         |                    | VL-VM 0.034     |                        |            |
|          |                   |         |                    | VM-CL <0.001    |                        |            |
|          |                   |         |                    | VL-CL <0.001    |                        |            |
| PSD length | Kruskal Wallis | 0.012   | 2                  | 275 PSD         | 4 mice                  | Dunn Sidak |
|          |                   |         |                    | 275 PSD         |                        |            |
|          |                   |         |                    | VL-VM 0.024     |                        |            |
|          |                   |         |                    | VM-CL 1.000     |                        |            |
|          |                   |         |                    | VL-CL 0.055     |                        |            |
| Number of Release sites | Kruskal Wallis | 0.667   | 2                  | 90 terminals    | 4 mice                  | Dunn Sidak |
|          |                   |         |                    | 90 terminals    |                        |            |
| Dendritic diameter | Kruskal Wallis | 0.080   | 2                  | 97 dendrites    | 4 mice                  | Dunn Sidak |
|          |                   |         |                    | 97 dendrites    |                        |            |
| 7C       | Protrusion surface vs terminal surface | Spearman | R=0.6146 P<0.001 | 44 protrusions | 4 mice                  |            |
|          |                   |         |                    | 44 protrusions  |                        |            |
| 7C       | Protrusion surface vs terminal surface | Spearman | R=0.7156 P<0.001 | 82 mitochondria | 4 mice                  |            |
|          |                   |         |                    | 82 mitochondria |                        |            |