SynBot: An open-source image analysis software for automated quantification of synapses

Justin T. Savage[1], Juan Ramirez[1], W. Christopher Risher[2], Yizhi Wang[3], Dolores Irala[4*], Cagla Eroglu[1,4,5,6,7*]

[1]Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA

[2]Department of Biomedical Sciences, Joan C. Edwards School of Medicine at Marshall University, Huntington, WV 25755, USA.

[3]Bradley Department of Electrical and Computer Engineering, Virginia Polytechnic Institute and State University, Arlington, VA 22203, USA

[4]Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA

[5]Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA

[6]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

[7]Lead contact.

*Correspondence: dolores.irala@duke.edu and cagla.eroglu@duke.edu

Keywords

image analysis, microscopy, synapse, FIJI, machine learning, immunofluorescence

Summary

The formation of precise numbers of neuronal connections, known as synapses,

is crucial for brain function. Therefore, synaptogenesis mechanisms have been one of the

main focuses of neuroscience. Immunohistochemistry is a common tool for visualizing

synapses. Thus, quantifying the numbers of synapses from light microscopy images

enables screening the impacts of experimental manipulations on synapse development.

Despite its utility, this approach is paired with low throughput analysis methods that are

challenging to learn and results are variable between experimenters, especially when

analyzing noisy images of brain tissue. We developed an open-source ImageJ-based

software, SynBot, to address these technical bottlenecks by automating the analysis.

SynBot incorporates the advanced algorithms ilastik and SynQuant for accurate

thresholding for synaptic puncta identification, and the code can easily be modified by

users. The use of this software will allow for rapid and reproducible screening of synaptic

phenotypes in healthy and diseased nervous systems.

Motivation

Light microscopy imaging of pre- and post-synaptic proteins from neurons in tissue

or *in vitro* allows for the effective identification of synaptic structures. Previous methods

for quantitative analysis of these images were time-consuming, required extensive user

training, and the source code could not be easily modified. Here, we describe SynBot, a

new open-source tool that automates the synapse quantification process, decreases the

requirement for user training, and allows for easy modifications to the code.

Introduction

Proper brain function relies on the correct wiring of neuronal networks[1–4]. Asymmetric neuron-neuron connections, known as synapses, are the fundamental cell biological units of neural circuits[5–7]. Importantly, synapse loss or dysfunction is a hallmark of many neurodevelopmental and neurodegenerative disorders[8–10]. Therefore, studying synapse connectivity is crucial to understanding how neuronal circuits are established during development, remodeled throughout life, and impacted by diseases.

Synapses are composed of two neuronal structures: the presynapse, located in the axon terminal, and the postsynapse, located on the dendrite. The presynapse contains specialized, neurotransmitter-filled, synaptic vesicles[6,11]. Some of these vesicles are docked at the presynaptic active zone and are fused with the membrane when an action potential reaches the synapse[11]. Neurotransmitters then diffuse into the extracellular space between the pre- and post-synapse, called the synaptic cleft[12]. Neurotransmitters in the synaptic cleft bind post-synaptic neurotransmitter receptors[13–15]. These receptors are transmembrane proteins that pass ions and/or recruit intracellular signaling partners to transduce a signal into the post-synaptic cell[7,13–15] and are anchored at the postsynapse through a scaffold of postsynaptic density proteins[16,17].

Synapses are dynamic structures that can be strengthened or lost due to changes in the inputs that neurons receive[18–20]. On the other hand, perturbations in genes that control synaptogenesis would also impact the number and organization of synaptic structures[21–23]. Non-neuronal cell types, such as astrocytes, microglia, and oligodendrocytes, also serve as critical regulators of synapse formation and elimination[24–28]. In particular, astrocytes, the major perisynaptic cells in the brain, strongly induce the

formation of excitatory and inhibitory synapses through direct contact with neuronal

processes or via the secretion of several synaptogenic proteins (reviewed in [1,29–31]).

The gold standard methods for interrogating the structure and function of synapses

are electron microscopy and electrophysiology, respectively. Electron microscopy (EM)

allows the experimenter to visualize the synapse with a high enough resolution (~2nm) to

resolve the pre- and post-synaptic compartments individually[32–34]. The characteristics of

presynaptic vesicles and postsynaptic densities are used to identify excitatory versus

inhibitory synapses[34–36]. An experimenter can count the number of opposing pre- and

post-synaptic sites in electron micrographs of samples from various conditions to

determine if these conditions alter the number of synaptic structures[34–36].

A common method for investigating synaptic function is the whole-cell patch-clamp

analysis of miniature postsynaptic currents. The frequency of miniature postsynaptic

currents provides information about the number of synapses a cell receives or the

probability of release at the presynaptic site. On the other hand, the amplitude of these

currents measures postsynaptic strength[37].

Electron microscopy and electrophysiology continue to provide high-resolution

structural and functional information about synaptic connectivity, but these techniques

have major limitations. First, they require extensive sample preparation and specialized

equipment, making them difficult to establish in a laboratory that does not specialize in

them. Second, they have very low throughput and sample only a small subset of synapses

or neurons, making them unsuited for screening multiple experimental conditions.

To address these limitations, higher-throughput methods for assessing synapse

numbers using immunostaining have proven useful[38,39]. These histological methods use

antibodies to label pairs of pre- and post-synaptic proteins that are specialized to distinct

synaptic subtypes. For example, presynaptic markers such as Vesicular Glutamate

transporter 1 (VGlut1), Vesicular Glutamate transporter 2 (VGlut2), or bassoon can be

paired with postsynaptic markers postsynaptic density protein 95 (PSD95) or Homer-1 to

label excitatory synapses[40–44]. On the other hand, the Vesicular GABA transporter

(VGAT), together with the postsynaptic Gephyrin, mark inhibitory synapses[41,45,46]. See

Verstraelen et al., 2020 for a detailed comparison of synaptic marker performance[47].

When these markers are imaged using light microscopy, due to the resolution limit (200-

300nm)[48], and the short (20-30nm)[44,49] distance between the pre- and post-synaptic

compartments, the color signals appear to overlap at synapses.

Therefore, synaptic structures can be quantified by counting the number of

colocalized pre- and post-synaptic markers using specialized image analysis software.

One of the first programs for performing this analysis is the Puncta Analyzer plugin for

ImageJ[38,] which has been widely used in neuroscience and yielded results that were then

validated through EM and electrophysiology[41,45,50,51].

A major limitation of Puncta Analyzer is that all analysis steps require user input,

which are time consuming and can be highly subjective. Thus, image analysis using

Puncta Analyzer is a lengthy process that requires extensive user training. In addition,

the source code for Puncta Analyzer is complex and difficult to edit to allow for

customization.

To circumvent these technical challenges, several alternative analysis pipelines

have been developed that automate portions of synapse counting. These include

methods to count synapses along a neurite (SynD and SynPAnal) [52,53], using additional

filtering steps to improve synapse detection from manual thresholding (SynapseJ[54]),

using automated thresholding algorithms in FIJI (Synapse Counter)[55], or development of

statistical thresholding algorithms (SynQuant)[56]. While these methods provided important

features beyond what is available in Puncta Analyzer, none have been as widely used as

Puncta Analyzer, which is well suited to quantify densely packed synapses like those

seen in the mouse brain tissues. To circumvent the limitations of Puncta Analyzer, here

we developed and tested a new open-source ImageJ-based synapse analysis software

we call SynBot, which is optimized not only for in vitro but also for high-noise *in vivo*

images and allows for a wide variety of options to tailor the analysis to the experimenter's

needs. We compared the accuracy and efficiency of SynBot with Puncta Analyzer using

simulated and experimental data which were previously validated by EM and

electrophysiology [57,58].

Results

Synapse labeling by immunohistochemistry

Synapse quantification relies on the use of immunohistochemistry to fluorescently label the pre- and post-synaptic compartments (**Figure 1A**). A standard immunostaining workflow is used, including 1) fixation and permeabilization of the sample, 2) application of primary antibodies against at least one pre-synaptic and one post-synaptic marker, 3) application of fluorescent secondary antibodies against the species of the primary antibodies used, and 4) image acquisition by fluorescence microscopy (**Figure 1B**). This protocol results in images where either excitatory (**Figure 1C**) or inhibitory (**Figure 1D**) synapses can be visualized. The details of the methods are explained in the Methods section and at protocols.io (https://www.protocols.io/view/synbot-protocols-3byl4qewjvo5/v2).

Developing an automated synapse quantification tool

Our first goal in developing SynBot was to enable the efficient quantification of large imaging datasets. SynBot combines many of the image processing and analysis steps that were performed for analysis with Puncta Analyzer into one automated workflow. Toward this aim, we first developed an ImageJ macro to automate the pre-processing steps that convert raw images into numerical representations, which can be used to calculate synaptic colocalizations (**Figure 2**). First, SynBot checks the image files to determine if they are z-stacks of confocal images or single images (either single optical section from a confocal or epifluorescence image). Then SynBot processes the z-stack files to produce max projections of each 1µm stack. This step is of particular use for *in vivo* synapse number analyses, which will be discussed in detail in a later section.

Second, all images are converted to the RGB format, the color format Image J uses.

Third, based on user selection, the FIJI Subtract Background, and Gaussian Blur Filter

functions are applied to remove noise from the image. The fourth step is the thresholding

of individual synaptic puncta to set a value for the background of the image and exclude

the pixels in the image that have an intensity value less than the background. A threshold

value can be determined in several different ways. The most common ones are manual

thresholding and ilastik-based automated thresholding. The users can also set the

threshold to a specified value. Fifth, the user decides whether to quantify synapses within

a region of interest or within the whole image. After entering these user inputs, the

program runs the FIJI Analyze Particles function to record the location and area of each

punctum in the region of interest of the thresholded image. This function records the X

and Y coordinates and area of each punctum for each color channel. Each punctum is

then compared to the puncta in the other channel of the image by approximating each

punctum to a circle or through a pixel-by-pixel approach discussed below.

SynBot analysis workflow

The files required for running SynBot and the installation instructions can be found

at https://github.com/Eroglu-Lab/Syn_Bot. There are also tutorials and instructional

videos on protocols.io (https://www.protocols.io/view/synbot-protocols-3byl4qewjvo5/v2).

Once the Syn_Bot.ijm macro is started, a selection menu is displayed to allow the users

to select their analysis parameters (**Figure 2**). The selection menu has 6 main sections:

1) Channels, 2) Preprocessing, 3) Thresholding, 4) ROI type, 5) Analysis type, and 6)

Experiment Directory. The detailed functions of each option on the SynBot selection menu

are defined in **Table S1**. Below, we summarize the utility of each section.

1) **Channels:** The fundamental objective of SynBot is to determine the overlap between different color channel objects (i.e., pre- or post-synaptic puncta). SynBot does this by first converting images into the red, green, and blue (RGB) format, which allows for consistent labeling of channels throughout the program. Users can select either the 2-channel option to analyze colocalization between the red and green channels or the 3-channel option to analyze colocalization between the red, green, and blue channels. If your images are in CMYK (cyan, magenta, yellow, and key (black)) format, or you would like to analyze red and blue or green and blue puncta colocalization, then there is also the "Pick channels" option. Z-stack images are max projected to produce individual projections that SynBot analyzes as separate images. For the experiments described below, every 3 Z-stacks were combined to produce a 1 um max projection. The number of stacks to project together can be adjusted by the user or set to 1 to allow individual processing of each optical section.

2) **Preprocessing:** Images can be preprocessed after RGB conversion to reduce noise or adjust the brightness. Noise reduction is performed by applying FIJI's subtract background plugin followed by a Gaussian blur filter to aid in object detection. This process removes some of the image background to aid in object detection (see https://imagej.net/plugins/rolling-ball-background-subtraction). Brightness adjustment changes the intensity values of each image such that there is an equal percentage of saturated pixels across images. As these methods modify the image data that SynBot will use for analysis, we advise users to apply them with caution and ensure they are producing accurate results. These modifications should also be consistent across groups of images that will be included in the same experiment.

3) Thresholding: One of the most challenging aspects of image analysis is thresholding, the process of distinguishing the foreground (i.e., synaptic puncta) of an image from the background. To discriminate between true synaptic puncta and the background noise is not trivial due to the small size of synaptic puncta and variability in staining and imaging parameters between users and microscopes. Therefore, here we include a number of possible options that SynBot users can utilize. The most popular of these options is manual thresholding, where the user selects a threshold value for each channel of each image, and SynBot runs the rest of the analysis. In that sense, this option is similar to Puncta Analyzer, the predecessor of SynBot. However, we designed Synbot to have several time-saving user-friendly features. For example, Synbot automatically displays each of the image channels to be thresholded, minimizing the time required for user input. However, the manual thresholding method requires extensive user training to use properly and necessitates a significant amount of hands-on user time.

To address the limitations of manual thresholding, we implemented several automated thresholding methods. First, we created a new ilastik-based[59] thresholding method, where a machine learning model is used to threshold each channel of each image. ilastik is an open-source program developed by Anna Kreshuk's lab at the European Molecular Biology Laboratory. ilastik is trained by the user in a small number of images (typically 3-5) to threshold by extracting a set of user-defined features from the image and then feeding these features into a random forest machine learning model. This model is then applied to the rest of the images in the data set to determine if a given pixel is part of the foreground or background of the image. As in all

thresholding methods, ilastik requires careful troubleshooting to ensure accurate

results. The training should be done for each experiment independently and should

not be used across datasets unless they are collected under identical conditions, such

as replicates of the same experiment performed by the same investigator. However,

using ilastik has a distinct advantage: it can be applied to a large number of images

without the need for further user input and only takes 5-15 minutes to generate the

trained model.

In addition to ilastik, we also integrated the SynQuant algorithm for thresholding

into SynBot. This method was developed by Wang et al. in 2020[56], to specifically

threshold synapses from immunofluorescence images through a statistical probability-

based approach. This is explained in detail in Wang et al., 2020 but in short, the

method assesses each potential synaptic object by comparing it to its neighboring

pixels based on fluorescence intensity, object size, and local contrast. The SynQuant

implementation within SynBot allows the user to adjust the following parameters: Z-

score threshold (a statistical measurement of how many standard deviations values

are from the mean of a population), minimum object size, maximum object size,

minimum object fill, maximum width to height ratio, and estimated noise standard

deviation. For most datasets, the noise standard deviation and Z-score threshold are

the only parameters that require adjustment. The SynQuant batch processing of

images through SynBot takes approximately 5-10 minutes for the size of data sets

presented here without any need for user input, so trying different parameters on the

same images is feasible and recommended to determine the most accurate conditions

for analyses.

We added another feature to Synbot to aid the reproducibility of results

between users. All the thresholding values are saved for each image analyzed.

Therefore, an analysis performed with SynBot can be efficiently reproduced later using

the "Threshold From File" option. This option asks the user to supply a CSV file

containing the threshold values for each channel of each image. This option does not

work for the ilastik or SynQuant thresholding options because these algorithms do not

use a set thresholding value, but they can already be easily reproduced since they are

unsupervised methods.

4) ROI Type: SynBot's colocalization analysis can be targeted to a region of interest

(ROI) within the input image. One common application is the restriction of the analysis

to the region around the soma of a cultured neuron, as in Figure 5. Alternatively, users

can implement their own complex ROIs by using the FIJI clear functions to remove

unwanted regions prior to running SynBot on the entire image. During the selection of

the ROI, the user is also asked to input a minimum and a maximum pixel size for each

channel puncta. The area of the ROI selected for each image is recorded and included

in the summary.csv output file.

5) Analysis Type: Puncta analyzer calculated colocalization based on a circular

approximation colocalization approach[38] (**Figure 3**). Therefore, we included this

option also in SynBot. With the circular-approximation analysis mode selected, the

coordinates and radius of each punctum for the channels to be analyzed are passed

through the Syn_Bot_Helper java plugin (packaged within https://github.com/Eroglu-

Lab/Syn_Bot). We used a Java plugin because it can perform the necessary

calculations much more efficiently than if they were done with ImageJ macro

language. These values for each punctum in the first channel are then compared to

each punctum in the second channel to detect colocalizations and calculate their area

using the geometry summarized in **Figure 3**. First, the distance between the centers

of the two circles is calculated (**Figure 3A**). If this distance is less than the sum of the

radii of the two circles, then they are counted as a colocalization, and the area of the

colocalization is calculated (**Figure 3B**). The basic idea of the area calculation is to

use the two intersection points of the circles along with the center of each circle to

define two triangles while also finding the areas of the sector of each circle between

the two intersection points. Subtracting the area of each triangle from the area of its

surrounding sector gives half of the overlapping area contributed by that punctum.

This can be done for both puncta to give the total area of the overlap (co-

localization)[60]. The coordinates and area of each colocalization are then stored and

added to the colocalized puncta count. This method would only work if the synaptic

puncta had high circularity, which can be determined using the Measure tool in FIJI

and is recorded for individual puncta by SynBot. In our experience, most synaptic

markers used in primary culture, or the mouse cortex are approximately circular. A

table of experimental circularity values for the antibodies used in this paper is provided

in Table S2. We also directly compared this circular approximation to the pixel-by-pixel

alternative and found nearly identical results for the types of images shown here

(Figure S3).

The circular-approximation method is too slow to practically analyze three

channel images and fails to correctly calculate colocalizations between non-circular

objects. To overcome these limitations, here we developed a pixel-overlap

colocalization method within SynBot (**Figure 3C**). Rather than approximating each

punctum to a circle, this analysis mode compares puncta in a pixel-by-pixel fashion

using the FIJI Image Calculator plugin's AND function. The AND function generates

a new image that contains pixels that overlap between the red punctum and a green

punctum. This new image can then be used to quantify co-localization with a third

channel (blue). The coordinate and area information for colocalized puncta is then

collected. Thus, this method is more accurate and versatile and runs more quickly

than the circular approximation method.

6) Experiment Directory: The final user input field for the SynBot selection menu is to

pick a folder that contains experimental images, hereafter called the experimental

directory. The directory folder should include at least one subfolder. The subfolder(s)

of the experimental directory should contain only the image files to be used by SynBot.

We often divide experimental groups between the subfolders of the experimental

directory, but this is not required and does not affect the analysis. It is also important

that image names contain only one "." character preceding their file extension. The

program uses this "." to separate the file extension from the image name and will not

run properly if there are multiple. Incorrect file naming can be especially problematic

with some image type conversion programs that name each image with both the old

and new extension (e.g., converting a Leica .lif file to .tif format can result in a name

like "image1.lif.tif").

<u>SynBot Outputs:</u>

A key feature of SynBot is that the users can easily check if the method is counting the

correct objects as colocalized puncta because after each image is analyzed, SynBot

creates and saves a feedback image within the Output folder using the name of the image

with "_colocs" appended to the end. This "_colocs" image shows the original input image

with white circular overlays at the center position of each recorded colocalization. New

SynBot users are strongly encouraged to check these images after running the macro

and adjust their thresholding parameters or retrain their ilastik model if they see too few

or too many calculated colocalizations that do not match what is evident by eye. The

thresholded images for each channel are also saved so the users can later validate

whether each channel was accurately thresholded.

The primary output of SynBot, which contains the numbers of colocalized puncta

per image, is the "Summary" csv file. This file contains the most commonly used summary

measurements for synapse counting, such as the total numbers of red, green (and blue

if triple colocalization is used), and the numbers of colocalized puncta from each image.

The file also includes the thresholds and the settings for minimal pixel size. To allow for

analysis of the cumulative properties of synaptic puncta, the macro also saves the X and

Y coordinates and area of each individual puncta for the red, green, blue, and colocalized

puncta into separate CSV files. These results are then ready for statistical analyses by

using other software such as R or GraphPad.

Quantification of synaptic colocalization by SynBot in simulated images

To validate SynBot's performance and compare the automated thresholding

methods that are integrated within SynBot, we tested SynBot on simulated synapse

images. We produced 20 simulated images with 667 red puncta, 666 green puncta, and

333 points where these puncta overlap to form a synapse. We then added a Gaussian

noise background to each image with a mean and standard deviation proportional to the

intensity histogram of the original image multiplied by 0.00, 0.25, 0.50, 0.75, or 1.00 to

simulate images with varying noise levels (**Figure 4A**). This produces a set of images

with no background (0.00), a background similar to what would be acquired in an

experiment (0.25-0.75), and an excessive level of background (1.00). We then ran SynBot

on these images using manual, ilastik, or SynQuant thresholding. Since the position of

these simulated synapses was known, we computed the recall (True Positive / (True

Positive + False Negative)) (**Figure 4B**) and precision (True Positive / (True Positive +

False Positive)) (**Figure 4C**) for each method on our simulated images. These metrics

are well suited to analyses where true positive, false positive, and false negative counts

are obtained but true negative counts are not well defined (every point on the image

without a synapse could be considered a true negative). We found that the recall and

precision values were similar for each method. However, the methods all had decreased

accuracy when the images contained excessive background. We found no significant

differences between the recall or precision obtained with these thresholding methods

when using a 2-way ANOVA at each noise level. Together these analyses indicate that

SynBot accurately counts synapses in realistic images containing low and high

background noise.

Quantification of astrocyte-induced synaptogenesis by SynBot _in vitro._

To test SynBot's utility and accuracy, we next used a glia-free cortical neuronal

culture system[61,62]. These neurons form few synapses when cultured alone; however,

astrocytes secrete proteins that strongly promote excitatory and inhibitory synapse

formation in these cultures[61,62]. The synaptogenic effects of astrocyte-conditioned media

(ACM) treatment are robust and promote a highly reproducible increase in the

colocalization of pre and postsynaptic puncta. This histological effect has been validated

by numerous studies using electron microscopy and electrophysiology[61,63–66]. Therefore,

to test the utility of SynBot and compare it to Puncta Analyzer, here we used ACM

treatment of purified cortical neuron cultures to induce synapse formation.

For these studies, neurons and astrocytes were individually prepared from

postnatal day 1 (P1) Sprague-Dawley rat pups. First, we dissected the cerebral cortex

and performed enzymatic digestion with papain and DNAse inhibitor for 45 minutes at

32C. To stop the enzymatic digestion, we treated the tissue with ovomucoid, followed up

by mechanical dissociation to obtain a single cell suspension (**Figure 5A**). This single cell

suspension was then used to isolate neurons or astrocytes.

As illustrated in **Figure 5B**, neurons were purified from the single cell suspensions

first through a series of negative panning steps to remove unwanted cell types in the

following manner: first using Baneiraea Simplicifolia Lectin 1, we removed macrophages,

second, using an anti-mouse secondary antibody-coated plate and an anti-rat secondary

antibody-coated plate we removed potential non-specific Fc-binding cells such as

microglia and cell debris. After the negative selection, we then incubated the cell

suspension on a positive panning petri dish coated with anti-L1CAM antibody. Neonatal

cortical neurons highly express L1CAM, which is absent from other cortical cell types,

such as astrocytes and oligodendrocytes. Both excitatory and inhibitory neurons of the

cortex are retained on the plate after incubation, and gentle washes are used to remove

unbound cells. Finally, the neurons were collected from the positive-panning plate by

gentle pipetting and pelleted for resuspension in neuronal growth media and plated on

glass coverslips which are coated with poly-D-lysine (PDL) and laminin at a density of 70

thousand cells per coverslip.

To isolate astrocytes, we plated the cortical single-cell suspension onto a PDL-

treated tissue culture flask. After 3 days *in vitro* (DIV3), we purified the astrocytes, which

are tightly attached to the flask, by vigorously shaking so less-adherent cells are removed.

To collect the ACM, we incubated the astrocytes with minimal media for 4 days to promote

protein secretion. We collected the ACM and concentrated it using centrifugal

concentrator tubes (**Figure 5C**). After protein quantification, we applied 50ug/ml or

100ug/ml ACM to the glia-free neuronal cultures at DIV8 and DIV 11 to induce excitatory

and inhibitory synapse formation, respectively (**Figure 5D**).

To label excitatory and inhibitory synapses in culture, we fixed the DIV 13 neuronal

cultures and immuno-stained the synapses with pre- and postsynaptic markers. To

identify excitatory synapses, we used the presynaptic active zone marker Bassoon

together with the excitatory postsynaptic marker Homer1 (**Figure 5E**). To visualize

inhibitory synapses, we used Bassoon in combination with the inhibitory postsynapse

marker Gephyrin (**Figure 5G**). For image acquisition, we took single focal plane images

using an inverted Olympus FV3000 confocal laser scanning microscope with a 60X oil

immersion objective. The images were taken in a 1024X1024 resolution with a 2X digital

zoom. To identify individual neurons, we used the DAPI channel, and we imaged only the

neuronal cell bodies at least two cell diameters distant from other neurons. We used this

strategy to avoid overlapping cells and to use an unbiased method to select the neurons

to image. We imaged at least 20 cells for each condition, and the laser power for each

channel was adjusted such that the signal for each channel was bright without many

saturated pixels.

To determine the efficiency of SynBot to detect pre- and postsynaptic

colocalization, we analyzed images from cortical neurons treated with ACM or cultured in

growth media only (named here as control) using manual, ilastik, and SynQuant

thresholding methods of SynBot. We compared these results to analyses done by its

predecessor Puncta Analyzer (**Figure 5E-H and S2**). To aid these comparisons, we

normalized the number of excitatory synapses between the ACM-treated neurons to the

control values. As expected, ACM induced a significant increase in synapse numbers

(~1.5-fold) with each of the analysis methods used (student's t test $p = 0.005$, $p = 0.004$,

$p = 0.010$, and $p = 0.0150$ for Puncta Analyzer, SynBot Manual, SynBot ilastik, and

SynBot SynQuant respectively) (**Figure 5F**). Similarly, we found a 2-fold increase in the

number of inhibitory synapses when neurons were treated with ACM compared to the

control (student's t-test $p < 0.001$ for each analysis method) (**Figure 5H**). These results

indicate that SynBot is equally efficient in detecting both types of synaptic contacts as the

Puncta Analyzer. Moreover, these results show that the ilastik and SynQuant automated

thresholding methods can be used to determine changes in synapse numbers.

To determine whether manual or automated thresholding affected the numbers of

puncta identified, we also compared the raw numbers of green (presynaptic marker), red

(postsynaptic marker), and colocalizations identified between the Puncta Analyzer,

SynBot Manual, SynBot ilastik, and SynBot SynQuant. When compared to the other

methods, SynBot ilastik consistently identified more green, red, and colocalized puncta

from the images, regardless of the antibodies used or the treatment conditions (**Figure**

S1). This result is consistent with ilastik's tendency to detect more individual objects (here

puncta) based on 37 different image features. In contrast, manual thresholding only uses

intensity cutoffs and minimal puncta size as features for detection. Two-way ANOVA tests

found significant differences in the raw number of colocalized synapses detected by each

of the analysis methods for the *in vitro* excitatory and inhibitory datasets shown in **Figure**

5. When the number of colocalized puncta detected was normalized to the appropriate

control conditions; however, there were no significant differences between the 4

thresholding methods. Together, these results show that SynBot, with its user-friendly

and timesaving features, efficiently detects excitatory and inhibitory synaptic contacts *in*

vitro.

Quantification of *in vivo* excitatory synapse numbers using SynBot

After validating the use of SynBot *in vitro*, we next tested its utility in quantifying

synapse numbers from mouse brain tissue sections. Immunohistochemistry of brain

tissue sections is a common method for synapse number quantification[40,41,45,46,57,62].

However, these analyses have the considerable added complexity of a 3-dimensional

tissue section that should be imaged. Moreover, thick tissue sections from the brain

inherently have more background signals, which can impair the accuracy of the analyses.

Finally, antibody penetration into the tissue sections is an important consideration while

optimizing synapse staining and imaging procedures. Before using SynBot or any other

method, the experimenters should optimize their staining and imaging procedures. The

optimized synapse staining and imaging procedure we have used can be found in the

methods   and   protocols.io   (https://www.protocols.io/view/synbot-protocols-

3byl4qewjvo5/v2).

To test SynBot's efficacy and accuracy in quantifying synapse numbers *in vivo*, we

next analyzed images that were previously acquired and reported in a study by Risher

and colleagues in 2018[57]. This paper showed that the neuronal

Thrombospondin/Gabapentin receptor α2δ-1 is crucial for proper excitatory synapse

formation in the developing mouse cortex[57,64,67]. Risher et al., 2018 showed that in α2δ-1

KO animals, there is a significant decrease in VGluT1/PSD95 synapse numbers

compared to WT controls[57]. Risher et al. validated these histological findings of impaired

synaptogenesis using electrophysiology and electron microscopy that also showed a

strong decrease in excitatory synapse function and number, respectively.

To perform these experiments, mice were transcardially perfused with 4%

paraformaldehyde, and brains were collected, frozen, and cryosectioned (**Figure 6A**).

Intracortical excitatory synapses were marked with the pre- and post-synaptic markers

specific for these connections, namely vesicular glutamate transporter 1 (VGluT1) and

postsynaptic density 95 (PSD95) (**Figure 6B**). Images were acquired using a Leica SP5

confocal microscope. A z-stack of 15 optical planes was acquired using a 0.34 um z-step

to image ~ 5 μm of the sample. Images were acquired such that confocal settings were

optimized on control conditions to minimize oversaturated pixels. For detailed procedures,

see the methods section and protocols.io (https://www.protocols.io/view/synbot-

protocols-3byl4qewjvo5/v2).

We used this well-validated data set to test SynBot's efficacy for quantifying

synapse numbers in mouse brain tissue images. We reanalyzed the P21 α2δ-1 WT and

KO images previously published in Risher et al., 2018 using the SynBot manual, ilastik,

and SynQuant thresholding methods and compared these to the results Risher et al.

obtained using Puncta Analyzer (**Figure 6C**). All 4 methods found a significant ~50%

decrease in the number of synapses in α2δ-1 KO animals when compared to WT

littermates (linear mixed effects model, p < 0.001 for each method). In our hands, a pre-

trained ilastik model implemented in SynBot or SynQuant automated thresholding was

able to perform as well as a highly trained manual user (**Figures 6C and S1)**. As we saw

with the *in vitro* datasets, there were significant differences between the raw numbers of

colocalized puncta detected by the 4 thresholding methods but not after normalization to

the WT control data (two-way ANOVA with p-value cutoff of 0.05). Quantification using

SynBot manual, ilastik, or SynQuant thresholding recapitulated the previously validated

finding that α2δ-1 KO mice have fewer intracortical synapses than WT littermates, which

was also previously confirmed by EM and electrophysiology[57]. This demonstrates that

SynBot can be successfully applied to brain tissue images and that the thresholding

methods packaged in SynBot can be used to compare different strategies quickly.

Altogether SynBot is a fast, reliable, user-friendly, and versatile software to study

synapse density *in vitro* and *in vivo*, overcoming previous constraints of Puncta Analyzer.

Discussion

Quantification of synapse numbers using immunohistochemistry is a favored technique for its ability to label synapses quickly and easily. However, this rapid experimental protocol is often paired with slow analysis methods that are difficult to learn and result in variability between users. SynBot overcomes many of the shortcomings of its predecessor by being easy to learn with built-in and online instructions and easy to use with a user interface that simplifies and streamlines user input. SynBot also allows unsupervised analyses through its automated methods. Importantly, SynBot collects all the data extracted from synaptic images by recording the position and area of each individual punctum from each image channel. These data can be used for other forms of analyses, such as the count and sizes of individual synaptic puncta or the spatial relationship in their distribution across the tissue imaged.

One of the most challenging aspects of analyzing these data is the subjective nature of the manual thresholding used in the Puncta Analyzer. The 9 thresholding modes available in SynBot address this by providing the user with simple approaches like a fixed threshold value, streamlined manual thresholding as well as the complex machine learning-based algorithm of ilastik and probability-based algorithm of SynQuant. We have found ilastik to be particularly suited to this workflow as a short training session in ilastik is sufficient to analyze images accurately and reproducibly without the need for further user input. The details about how to train ilastik for synaptic puncta detection can be found in the methods section and protocols.io. When used in the manual mode, SynBot also automatically saves the threshold values applied to each image with the output data, allowing for rapid replication of the analysis by other users.

The advantage of implementing SynBot in the ImageJ macro language is the ease

with which it can be changed by users to apply to a wider variety of experimental

questions. All the source code for the macro is available on our lab GitHub site

(https://github.com/Eroglu-Lab/Syn_Bot) and can be edited within FIJI itself without the

need for software development programs or in-depth computer science experience.

There were only a few parts of SynBot that could not be implemented in ImageJ macro

language (circular-approximation colocalization, some dialog menus, ilastik and

SynQuant integration) and required Java programming. These sections are stored in the

separate   GitHub   repositories   ilastik4ij_Syn_Bot   (https://github.com/Eroglu-

Lab/ilastik4ij_Syn_Bot) and (https://github.com/freemanwyz/SynQuantSimple), which are

freely available for download and can be edited by users with experience in Java

programming.

Another benefit of SynBot over previous analysis programs is its speed. When

analyzing the 100 simulated images shown in Figure 4, for example, SynBot analysis took

24 minutes for manual thresholding, 10 minutes for training and 50 minutes for ilastik

thresholding, and 4 minutes with SynQuant thresholding. Unlike manual thresholding, the

time spent while ilastik or SynQuant run requires no user input. The speed of SynQuant

makes it well-suited for running multiple times using different parameters, making it easy

to troubleshoot and optimize accuracy for a given data set.

With all of these features, researchers will be able to rapidly screen experimental

conditions that alter synapse numbers and can move on to in-depth structural and

mechanistic investigations with other methods such as electrophysiology, super-

resolution or electron microscopy. Therefore, SynBot will aid in answering many

outstanding questions related to synapse development and maintenance.

Limitations of the Study

There are a few key conditions necessary for SynBot to be successfully applied.

Firstly, the signals being analyzed need to be punctate rather than diffuse since SynBot

performs object-based colocalizations and must be able to identify discrete objects.

Similarly, objects within the images must be non-overlapping to be independently

counted. This object-based colocalization is most appropriate when the position of

synaptic compartments is changing to alter the number of structural synapses (seen as

colocalizations). If the abundance of a synaptic marker or its association with synapses

is changing, intensity measurements or pixel-wise colocalization on unthresholded raw

images may be more appropriate (see https://imagej.net/imaging/colocalization-analysis

for further discussion of different colocalization methods).

SynBot uses synaptic puncta position over raw intensity. Thus, we can use

thresholding methods such as ilastik and SynQuant that apply different values to each

image to separate the foreground from the background. However, if needed SynBot can

also apply the "Fixed Value" thresholding, which allows users to apply the same exact

threshold value to all images. An important limitation of this fixed thresholding method is

that it would only be useful for datasets with images containing minimal background noise,

such as *in vitro* synapse staining.

While theoretically possible, in practice staining intensities and background may

vary even within a single experiment enough to impair the use of fixed values. We strongly

recommend using methods like ilastik or SynQuant instead because they have the

flexibility to adjust to the varying image brightness. In particular SynQuant thresholds

images using a statistical method that provides consistency between different images.

Thus, these automated methods make these analyses much more accessible for

untrained new users than manual thresholding.

SynBot relies on RGB image conversion to keep track of the different image color

channels. This limits SynBot analysis to a maximum of 3 colors and converts each image

channel to an 8-bit format. This reduction in bit-depth from the 16-bit images acquired by

many modern microscopes reduces the possible thresholding values from 65,536 in a 16-

bit image to 256 in an 8-bit image. For the synapse analysis experiments shown here,

256 possible values is adequate for thresholding, but certain analyses may benefit from

dividing the image intensity range into a greater number of bins. It should be noted that

SynBot has no limitations for image resolution (the number of pixels representing a given

area of space) and that this bit-depth conversion has no impact on resolution.

SynBot only works on single optical sections or Z-stack projections and is unable

to fully incorporate 3-dimensional synapse structures, as is done in other software such

as Imaris. Despite these caveats, SynBot is widely applicable to any object-based

colocalization analysis for synapses or any other set of imaging markers. SynBot can

tailor its analysis to many experimental questions. However, for neurite tracing synapse

analysis or 3D constructions alternative methods should be used[52,53]. Similarly, if the

user needs to analyze multiple regions from an image, they should perform repetitive

analysis to follow that experimental design.

It is also important to note that the accuracy of synapse number analyses is heavily

dependent on the quality of staining and imaging. Moreover, proper markers should be

used to label synapses. Some common mistakes are to use an axonal or dendritic marker,

such as GAD65 or GAD67 (for inhibitory axons) or MAP2 (for neuronal dendrites), as one

of the synaptic compartments. These kinds of markers are often extrasynaptically

localized and widely distributed, thus yield co-localization with other markers even if they

were not at an actual synapse.

Similarly, SynBot requires careful troubleshooting, even when using fully-

automated thresholding methods. Users should always visually inspect the counted

synapses using the "colocs" output images and verify the thresholding performance by

viewing the "red_thresholded" and "green_thresholded" output images. It is also advisable

to include control conditions whenever possible, such as perturbations known to change

synapse numbers. As with any image analysis method, improper use can lead to

misleading results.

Acknowledgments:

We thank Christabel Tan, M. Pia Rodriguez-Salazar, Dr. Oluwadamilola Lawal, Nicholas Brose, Gabrielle Sejourne, Kavya Raghunathan and other Eroglu lab members for critical feedback about the manuscript. This work was supported NIH R01 funding AG059409, NS102237, and BRAIN Initiative funding U19NS123719 and a Chan Zuckerberg Initiative, Neurodegeneration Challenge Network Collaborative grant (2018-191999 and DAF2021-237435) and by the joint efforts of MJFF and the Aligning Science Across Parkinson's (ASAP) initiative. MJFF administers the grant [ASAP-020607 to CE] on behalf of ASAP and itself Michael J Fox Foundation. J.T.S. was supported by NIH funding F31NS134252. D.I. was supported by The Pew Latin American Fellows Program in the Biomedical Sciences, Foerster-Bernstein Post-Doctoral Fellowship Program for Women in STEM, and the Holland-Trice Scholars Award for high risk/high impact discovery in research in Basic Brain Science and Brain Diseases. JJR was supported by NIH funding NS102237-S1 and F31NS125985. YW was supported by NIH funding R01MH110504. WCR was supported by NIH funding NIH R15MH126345. Dr. Cagla Eroglu is an HHMI Investigator. The cartoon of a synapse in Figure 1 and a diagram of mouse brain tissue staining in Figure 5 were created using BioRender.com.

IP rights notice

Author Contributions

Conceptualization, J.T.S. and C.E.; Software, J.T.S., J.R., Y.W.; Investigation, J.R., W.C.R., and D.I.; Writing – Original Draft, J.T.S., J.R., D.I., and C.E.; Writing – Review & Editing, J.T.S., J.R., D.I., and C.E.

Declaration of Interests

The authors declare no competing interests.

Figure Legends:

Figure 1: Labeling of synapses as apposing colocalization of pre- and post-synaptic markers:

A) Illustration of excitatory and inhibitory synaptic structures. Presynaptic markers used in this paper are shown in green and postsynaptic markers are shown in magenta.

B) Synaptic staining workflow.

1. After fixation with 37°C PFA 4%, the neurons are permeabilized and non-specific epitopes are blocked in antibody buffer containing 50% NGS and 0.2% Triton X-100.

2. Neurons are incubated with primary antibodies targeting pre- and post-synaptic compartments of excitatory or inhibitory synapses.

3. After overnight incubation with primary antibodies, the neurons are washed with PBS.

4. Neurons are incubated with secondary antibodies conjugated to Alexa fluorophores.

5. After 2 hours of room temperature incubation with secondary antibodies, neurons are again washed with PBS and the neuronal nuclei are stained with DAPI.

6. Coverslips are mounted on glass slides using a medium that protects from bleaching of Alexa fluorophores for imaging.

   For details, check the methods section and protocols.io.

C) Representative image of excitatory synapses made onto a cultured cortical neuron stained with Homer1, Bassoon, and Vglut1. The scale bar in the larger image represents 20 μm, and the scale bar in the smaller image represents 5 μm.

D) Representative image of inhibitory synapses made onto a cultured cortical neuron stained with Gephyrin, Bassoon, and VGAT. The scale bar in the larger image represents 20 μm, and the scale bar in the smaller image represents 5 μm.

Figure 2: Workflow for SynBot synapse quantification

A) The SynBot selection menu serves as the primary point of user input to adjust options for synapse analysis.

B) The main steps in the SynBot colocalization analysis:

1) Images are loaded into the software and converted to RGB.

2) Images are denoised using the FIJI Subtract Background and Gaussian Blur Filter functions.

3) Each image channel is thresholded to separate synaptic puncta from the background.

4) A region of interest (ROI) for colocalization quantification can be selected
(yellow dashed-line).

5-6) Red and green puncta are detected by FIJI's Analyze Particles function and
counted.

7) Colocalization between red and green puncta are calculated. See Figure 3.

8) The colocalized puncta are overlayed on the original image, and the data are
saved as a .csv file.

The scale bar in the larger image represents 20 μm, and the scale bar in the
smaller image represents 5 μm.

Figure 3: Detection and calculation of puncta colocalization

A) SynBot detects colocalizations between synaptic puncta by first calculating the
distance between the centers of the puncta and then determining if that distance
is less than the sum of the radii of the puncta. If this distance is less than the sum
of the radii, the colocalization is recorded and the area of colocalization is
calculated.

B) SynBot runs the following calculations for the area of colocalization area.

1) Calculate the distance between the center of the first puncta and the line of
intersection.

2) Calculate the distances from the midpoint of the distance between the two
puncta centers (d) to the intersection of the edges of the two puncta.

3) Calculates the area of the sector of the first puncta between the two intersection

points.

4) Calculates the area of the sector for the second puncta.

5) Calculates the area of the rectangle formed by the two centers and intersection

points; the sum of the two triangular portions of the sectors that are not part of the

overlap.

6) The general form for the area of colocalization is the sum of the two sectors

minus the two triangular portions outside of the overlap.

C) Pictorial representation of the pixel-overlap analysis mode where FIJI's image

calculator is used to identify pixels that are part of both the red and green channel for a

2-channel colocalization or part of the red, green, and blue channels for a 3-channel

colocalization. Scale bar represents 1 µm.

Figure 4: SynBot quantifies simulated synapse data with high recall and precision.

A) Simulated synapse images with differing levels of gaussian noise background.

Synapses were copied from VGlut1-PSD95 a2d1 WT and KO images from figure

6 and then pasted onto Gaussian noise background with a mean and standard

deviation proportional to the same values in the original image multiplied by the

given noise level (0.00, 0.25, 0.50, 0.75, 1.00). Error bar represents 20um.

B) Recall (True Positive / (True Positive/ False Negative)) obtained using SynBot with

manual, ilastik, and SynQuant thresholding methods. 20 images were analyzed at

each noise level. Error bars represent 1 standard error of the mean.

C) Precision (True Positive / (True Positive/ False Negative)) obtained using SynBot with manual, ilastik, and SynQuant thresholding methods. 20 images were analyzed at each noise level. Error bars represent 1 standard error of the mean.

Figure 5: SynBot quantifies astrocyte-induced synapse formation *in vitro.*

A) Scheme of single cell suspension generation from P1 rat pup cortices.

B) Scheme of neuronal isolation by immunopanning first through a series of negative panning steps and then through positive panning with Anti-L1CAM antibody.

C) Scheme of astrocyte purification and astrocyte-conditioned media (ACM) preparation.

D) Timeline of neuronal feeding schedule and ACM treatment.

E) Representative images (in RGB) of excitatory synaptic puncta stained with presynaptic marker Bassoon and postsynaptic marker Homer1. Scale: 15 µm for full image and 5 µm for zoom in. Yellow dashed line represents the region of interest analyzed. White dots represent counted synapses.

F) Example of quantification of excitatory synaptic colocalization between control and ACM using the Puncta Analyzer, SynBot manual thresholding, SynBot ilastik, or SynBot SynQuant thresholding. Student's T-test, 20 neurons per treatment. Error bars represent 1 standard error of the mean.

G) Representative images (in RGB) of inhibitory synaptic puncta stained with presynaptic marker Bassoon and postsynaptic marker Gephyrin. Scale: 15 µm for full image and 5 µm for zoom-in. Yellow dashed line represents the region of interest analyzed. White dots represent counted synapses.

H) Example of quantification of inhibitory synaptic colocalization between control and ACM using the Puncta Analyzer, SynBot manual thresholding, SynBot ilastik thresholding, or SynBot SynQuant thresholding. Student's T-test, 20 neurons per treatment. Error bars represent 1 standard error of the mean.

Figure 6: SynBot quantifies reduced α2δ-1 KO synapse numbers *in vivo*.

A) Workflow for harvesting mouse brain tissue and preparing it for synapse imaging.

B) Representative images of synapses from layer 2/3 of primary visual cortex in α2δ-1 wild type vs. α2δ-1 knockout mice at postnatal day 21. Magenta = VGluT1; Green = PSD95. Synapses identified by each thresholding method are marked by small white dots. Scale bar represents 5 μm.

C) Quantification of VGluT1-PSD95 colocalization using the Puncta Analyzer, SynBot manual thresholding, SynBot ilastik thresholding, or SynBot SynQuant thresholding. Colocalized puncta counts were normalized to the WT average for each experimental pair. A linear mixed-effects model was used to account for multiple images per animal. Mouse averages are shown as large black dots (N = 3 mice per condition) with individual images shown as small gray dots. Error bars represent 1 standard error of the mean.

Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Cagla Eroglu (cagla.eroglu@duke.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Microscopy data and associated synapse counts reported in this paper are available on Zenodo at the following DOI: 10.5281/zenodo.12191805

- All original code has been deposited at our lab Github site (https://github.com/Eroglu-Lab/Syn_Bot) and is publicly available on Zenodo at the following DOI: 10.5281/zenodo.12192447

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Mice and rats were used for experiments as specified in the text and figure legends. All mice and rats were used in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Duke Division of Laboratory Animal Resources (DLAR)

oversight (IACUC Protocol Numbers A147-17-06 and A117-20-05). All mice and rats were

housed under typical day/night conditions of 12-hour cycles. For all experiments, age,

and sex-matched mice were randomly assigned to experimental groups based on

genotypes. The primary rat neurons and astrocytes were isolated from wildtype Crl:

CD(SD) Sprague-Dawley rats from Charles River Laboratories (RRID: RGD_734476).

METHOD DETAILS

In vitro Synapse Assay with Astrocyte Conditioned Media

Cortical tissue digestion

Primary rat astrocytes and neurons were isolated from postnatal day 1 (P1) rat

pups. Pups were rapidly decapitated, and brains dissected out and placed into a petri

dish of PBS. Brains were then micro-dissected to isolate the cerebral cortex, and the

cortices were chopped into pieces approximately 1 cubic millimeter in volume. A transfer

pipet was then cut where it began to taper to create a larger opening. This cut pipet was

then used to suck up the cortex chunks, and tissue was transferred to a tube of 7.5

units/mL papain digestion solution (Worthington, Cat# LK003178) by gently tapping on

the pipette without depressing it. Cortices were then digested for 45 minutes at 33°C with

a brief swirling at the 15- and 30-minute points. The digestion solution is then aspirated

off and the tissue is resuspended in a 2 mg/ml trypsin inhibitor ovomucoid solution (Lo

Ovo) (Worthington, Cat# LS003083). Cells were then spun in a centrifuge at 300g for 11

minutes at room temperature. The supernatant was then removed, and the cells were

resuspended in a 4 mg/ml Ovomucoid solution (Hi Ovo). Cells were then spun again at

300g for 11 minutes and the supernatant removed. The resulting cells were then either

used for isolating neurons or astrocytes according to the following sections.

Neuronal Isolation by Immunopanning

The pelleted cells from the cortical tissue digestion were resuspended in Panning

Buffer (0.02% BSA with 0.5 ug/ml insulin in DPBS with calcium, magnesium, glucose,

and pyruvate (Thermo Fisher, Cat# 14287080)). The cells were then filtered through a

20µm nylon mesh (Elko Filtering, Cat# 03-20/14) to remove clumps. These filtered cells

were then added to a petri dish which had been treated with Griffonia Simplicifolia Lectin

I (Vector Laboratories, Cat# L-1100) for 1 hour at room temperature prior to adding the

cells. The cells were incubated on this lectin plate for 10 minutes at room temperature.

The plate was then forcefully shaken to resuspend any loosely adhered cells. The solution

in the lectin plate was then transferred to a second lectin plate for a 15-minute incubation

at room temperature. The second lectin plate was then forcefully shaken, and the solution

transferred to a plate treated with an L1CAM antibody (α-L1) (Developmental Studies

Hybridoma Bank, Cat# ASCS4) which binds to neurons. The cells were incubated on the

α-L1 plate for 45 minutes at room temperature. The media was then gently removed from

the α-L1 plate and the plate was washed 5 times with panning buffer. Cells bound to the

α-L1 plate were then resuspended in the panning buffer and collected in a conical tube.

The cells were then spun for 11 minutes at 300g. The supernatant was removed, and the

cells were resuspended in neuronal growth media (NGM: Neurobasal (Gibco, Cat#

21103049), B27 supplement (Gibco, Cat# 17504044), 2 mM L-Glutamine (Gibco, Cat#

25030-081), 100 U/mL Pen/Strep (Gibco, Cat# 15140), 1 mM sodium pyruvate (Gibco,

Cat# 11360-070), 4.2 µg/mL Forskolin (Sigma, Cat# F6886), 50 ng/mL BDNF

(PeproTech, Cat# 450-02), and 10 ng/mL CNTF (PeproTech, Cat# 450-13)). The cells

were then counted, and plated at a low density of 60,000 cells each onto poly-D-lysine-

treated (Sigma, Cat# P6407) and mouse laminin (Cultrex, Cat# 3400-010-01) treated

glass coverslips in a 24-well plate with neuronal growth media. Cells were then kept in a

tissue culture incubator at 37°C with 10% CO_2.

Astrocyte Isolation and ACM Production

The pelleted cells from the cortical tissue digestion were resuspended in astrocyte

growth media (AGM: DMEM (GIBCO, Cat# 11960), 10% FBS (Thermo Fisher, Cat# 10-

437-028), 10 mM, hydrocortisone (Sigma, Cat# H-0888) , 100 U/mL Pen/Strep, 2 mM L-

Glutamine, 5 mg/mL Insulin (Sigma, Cat# 11882), 1 mM Sodium Pyruvate, 5 mg/mL N-

Acetyl-L-cysteine (Sigma, Cat# A8199)). The cells were then filtered through a 20µm

nylon mesh to remove cell clumps. The filtered cells were then spun for 9 minutes at 300g.

The supernatant was then removed, and the cells were resuspended in AGM and

counted. 15 million cells were then plated in a T-75 flask with a non-aerating top. Cells

were then kept in a tissue culture incubator at 37°C with 10% CO_2 with the cap slightly

unscrewed to allow airflow.

On DIV3, astrocyte flasks were washed 3 times with DPBS without calcium or

magnesium (Gibco, Cat# 14190144). After the third wash, 20 mL of DPBS without calcium

or magnesium was added and the flask was vigorously shaken to remove loosely

adherent cells. This shake-off process significantly enriches for astrocytes. Media was

then replaced with AGM and flasks were returned to their incubator. On DIV 5, astrocytes

were treated with 2.43 mg/ml Cytosine arabinoside (AraC) (Sigma, Cat# C1768) through

a full media change. On DIV 7 astrocytes were passaged using 0.05% Trypsin-EDTA. 3

million cells each were then plated into 10 cm culture dishes. On DIV 9, astrocytes were

switched to a minimal media (Neurobasal medium minus phenol red (Gibco, Cat#

12348017), 100 U/mL pen/strep, 2mM L-glutamine, and 1mM sodium pyruvate) for

astrocyte conditioning. Astrocytes were then cultured for 5 days without any media

change, and on DIV 14, the ACM was collected. The ACM was then centrifuged for 5

minutes at 1,100g to pellet cellular debris. The supernatant was concentrated by

centrifugation for 1 hour at 3220 g at 4°C in a 5kDa Cutoff Vivaspin tube (Sartorius, Cat

VS2012). ACM was aliquoted into low protein binding tubes (~100ul per aliquot), flash-

frozen in liquid nitrogen, and stored at -80°C until use. An aliquot of each batch of ACM

was thawed and used to measure the protein content using the Pierce BCA protein assay

kit (Thermo Fisher, Cat# 23225).

Neuron Feeding and ACM Treatment

On DIV2, neurons were treated with 2.43 mg/ml AraC through a half-media

change. A full media change was then performed 24 hours later (DIV3) to remove the

AraC. Neurons were then given another half-media change on DIV6. Neurons were then

fed with ACM on DIV8 and DIV 11 by adding either 50 mg/ml ACM for excitatory synapses

or 100 mg/ml ACM for inhibitory synapses in a half media change.

Neuron Staining and Imaging

On DIV 13, neurons were fixed with 4% paraformaldehyde (Electron Microscopy

Sciences, Cat# 15710) in PBS. Cells were then washed 3 times with PBS and then

blocked for 30 minutes at room temperature with antibody blocking buffer (150 mM NaCl,

50 mM Tris-Base, 1% BSA, 100 mM L-lysine 50% normal goat serum (Thermo Fisher,

Cat# 01-6201), 0.2% Triton (Roche, Cat# 11332481001). The blocking buffer was then

removed, and cells were incubated with primary antibodies against the pre- and post-

synaptic markers of interest overnight at 4°C in antibody incubation buffer (150 mM NaCl,

50 mM Tris-Base (VWR, Cat# 101174-856), 1% BSA, 100 mM L-lysine, 10% normal goat

serum). The following day cells were washed 3 times with PBS and incubated in

fluorescent secondary antibodies in antibody incubation buffer for 2 hours at room

temperature protected from light. Cells were then washed with PBS 3 times and incubated

with 1:10,000 DAPI (Invitrogen, Cat#D1306) for 5 minutes. Cells were then washed 3

times with PBS and mounted onto coverslips with homemade mounting media (20mM

Tris pH 8.0, 90% Glycerol, 0.5% N-propyl gallate) and sealed with nail polish.

Single focal plane images were taken using an inverted Olympus FV3000 confocal

laser scanning microscope with a 60X oil immersion objective. The images were taken in

a 1024X1024 resolution with a 2X digital zoom. For the picture acquisition, the DAPI

channel was used to select neuronal cell bodies distant from other neurons to avoid

overlap of cell soma and to do a blind selection of the neurons. DAPI positive nuclei with

abnormal morphology were excluded for the analysis. 20 cells for each condition were

selected for the analysis. Images were also saved as .bmp extension with only Bassoon

(green) and Gephyrin (red channel) for the synaptic analysis.

Mouse Brain Tissue Synapse Assay

Mouse perfusion and cryosectioning

P21 α2δ-1 WT and KO mice were euthanized by intraperitoneal injection of 0.6mL

of 12.5 mg/mL 2,2,2-tribromoethanol (Avertin) (Sigma, Cat# T48402-25G) followed by

exsanguination through transcardially perfusion of tris-buffered saline (TBS: 137mM

NaCl, 2.68 mM KCl, 24.8 mM Tris-base (Thermo Fisher, Cat# J75825.A7)). Following

TBS perfusion, animals were perfused with warm, 4% paraformaldehyde (PFA) in TBS

solution. Brains were then removed and post-fixed in 4% PFA overnight at 4°C. After post-

fixation, brains were washed 3 times with TBS and then incubated in 30% sucrose in TBS

for 48-72 hours. Brains are fully cryopreserved once they sink to the bottom of their

container in the 30% sucrose. Brains were then placed in cryomolds and frozen in 50%

Optimal Cutting Temperature Compound (OCT) (Tissue Tek, Cat# 4583) in TBS on dry

ice. Brains were then kept at -80°C until sectioning. Brains were cryosectioned to a

thickness of 15-40 µm and stored in 50% glycerol (Acros Organics, Cat# 15892-0010) in

TBS at -20°C.

Tissue Section Synapse Staining and Imaging

Three tissue sections per animal were washed 3 times for ten minutes with 0.2%

TritonX-100 in TBS (TBST) on a shaker at room temperature. Sections were then blocked

with 5% normal goat serum in TBST for 1 hour at room temperature on a shaker. Sections

were then stained with primary antibodies for the pre and postsynaptic markers of interest

overnight at 4°C on a shaker. Sections were then washed 3 times in TBST for 10 minutes

and incubated with fluorescent secondary antibodies for 2 hours at room temperature on

a shaker protected from light. After secondary incubation, sections were washed 3 times

with TBST for 10 minutes. After washing, sections were mounted onto microscope slides

and sealed with nail polish.

Stained tissue is ready to image after nail polish is dried and should be imaged

within 48 hours of secondary staining for most accurate results. Images were acquired

using a Leica SP5 confocal microscope with a 63X oil immersion objective. For 1024 X

1024 images, a 1.64X digital zoom was used to achieve an XY resolution of 0.126 um X

0.126 µm pixel size and a z-stack of 15 optical planes was acquired using a 0.34 µm z-

step to image ~ 5 µm of the sample. In general, images were acquired such that confocal

settings were optimized on control conditions with the goal of minimizing oversaturated

pixels but maintaining real synaptic puncta.

Image Analysis

Synapse Quantification with Puncta Analyzer

In vitro synapse assay images were analyzed using Puncta Analyer[38] by first

converting the images to the RGB format and then running the Puncta Analyzer plugin on

each image. A circular ROI was applied to each image with a radius of 301 pixels around

the cell body of the imaged neuron. A threshold for each image was chosen to minimize

the background while preserving the signal.

In vivo, synapse assay images were Z-projected such that every 3 optical planes

were projected to produce 5 max-projections corresponding to 1 µm, per 15-image stack

covering the 5 µm. Images were then analyzed with the Puncta Analyzer plugin using

default settings. A threshold for each image was chosen by users to minimize the

background while preserving the signal.

Synapse Quantification with SynBot

Images were analyzed with the SynBot macro using the default settings for the

manual thresholding method. A threshold for each image was chosen to minimize the

background while preserving the signal. Threshold performance was checked visually

using the "_colocs" image SynBot creates that displays which puncta were counted as

synapses in each image.

For the ilastik thresholding method, images were first preprocessed into individual

color channels using the extract_channels macro (available at https://github.com/Eroglu-

Lab/Syn_Bot). These single-channel images were then used to train ilastik projects for

each image channel. Each ilastik project was trained using the pixel classification mode

in ilastik. A random subset of images from the target color channel was chosen for the

training set. For both *in vitro* and *in vivo* synapse analysis, we used all the image features

available. We then annotated approximately 20 synapses as label 1 and marked several

regions of the background as label 2 on the first image. We then used the live update

feature to view the classifications of each pixel and added additional annotations to the

first image and subsequent images as necessary. We then saved the ilastik project files

and used these for the SynBot ilastik thresholding method (all ilastik project files used for

this paper are available at https://github.com/Eroglu-Lab/Syn_Bot).

For the SynQuant thresholding method, images were analyzed using the

SynQuant batch thresholding option and SynBot noise reduction. The following SynQuant

parameters were used: Simulated data: Z-score threshold = 10, minimum object size =10,

maximum object size = 100, minimum object fill = 0.5, maximum width to height ratio = 4,

Z axis multiplier = 1, and estimated noise standard deviation = 12. *In vitro* data: Z-score

threshold = 10, minimum object size =10, maximum object size = 100, minimum object

fill = 0.5, maximum width to height ratio = 4, Z axis multiplier = 1, and estimated noise

standard deviation = 20. *In vivo* data: Z-score threshold = 10, minimum object size =10,

maximum object size = 100, minimum object fill = 0.5, maximum width to height ratio = 4,

Z axis multiplier = 1, and estimated noise standard deviation = 12.

Creation of Simulated Synapse Images

To produce these simulated images, we first used a set of 20 real VGlut1-PSD95 synapse

images from Risher et. al., 2019. We split the two image channels and then measured

the pixel intensity histogram from these images. We generated a Gaussian noise

background for each image by multiplying the mean and standard deviation of the original

image's histogram by a multiplier (0.00, 0.25, 0.50, 0.75, or 1.00). Since the pixel intensity

histogram includes the true signal as well as the background, the 1.00 multiplier will

produce an image with a higher background than that of the original image. This makes

the range of 0 to 1 for our noise multiplier represent images with no background at (0.00),

moderate background comparable to experimental data (0.25-0.75), and high

background beyond what would be acceptable for analysis (1.00). We next took 10

synaptic puncta from each channel of the original image and pasted these onto the

background images we generated. Synaptic puncta were pasted into 1000 possible

positions such that 334 positions had a red puncta only, 333 positions had a green puncta

only, and 333 positions had a red puncta and a green puncta at the same location. Note

that the locations for pasting these synaptic puncta were defined by the top left corner of

their bounding box. This resulted in varying levels of overlap similar to real synaptic

imaging data and explains the precision and recall of SynBot being below 1.0 since many

of these synapses were no longer overlapping after being thresholded. The end product

of this code was 100 total images with 20 images having each of the 5 background levels.

The ImageJ macro code used for generating these simulated images is available at

https://github.com/Eroglu-Lab/Syn_Bot).

QUANTIFICATION AND STATISTICAL ANALYSES

SynBot was used to quantify synapse numbers in the experiments shown.

Subsequent statistical analysis was performed with the R statistical software (R Core

Team). Student's t-tests were used to test for significance between conditions for the *in*

vitro experiments, and linear mixed-effects models were used (R package nlme, R Core

Team) to test the significance of *in vivo* experiments and account for the nested structure

of the data (multiple z-stacks per image and multiple images per animal). Statistical

details of each experiment can be found in the figure legends. For the cell culture

experiments, each n represents a neuron that was imaged. For mouse tissue sections

experiments, each n represents the average synapse density of a mouse that was

included in the experiment. For each mouse, at least 15 max-projection images

corresponding to 3 independent z-stacks were used. The full R code for performing the

statistical analyses and producing the plots in this paper is available at

https://github.com/Eroglu-Lab/Syn_Bot.

References

1.   Farhy-Tselnicker, I. & Allen, N. J. Astrocytes, neurons, synapses: a tripartite view on
cortical circuit development. *Neural Dev* **13**, (2018).

2.   Cadwell, C. R., Bhaduri, A., Mostajo-Radji, M. A., Keefe, M. G. & Nowakowski, T. J.
Development and Arealization of the Cerebral Cortex. *Neuron* **103**, 980–1004 (2019).

3.   Pan, Y. & Monje, M. Activity Shapes Neural Circuit Form and Function: A Historical
Perspective. *Journal of Neuroscience* **40**, 944–954 (2020).

4.   Luo, L. Architectures of Neuronal Circuits. *Science* **373**, eabg7285 (2021).

5.   Batool, S. *et al.* Synapse formation: From cellular and molecular mechanisms to
neurodevelopmental and neurodegenerative disorders. *J Neurophysiol* **121**, 1381–1397
(2019).

6.   Rizalar, F. S., Roosen, D. A. & Haucke, V. A Presynaptic Perspective on Transport and
Assembly Mechanisms for Synapse Formation. *Neuron* **109**, 27–41 (2021).

7.   Groc, L. & Choquet, D. Linking glutamate receptor movements and synapse function.
Science (1979) **368**, (2020).

8.   Newschaffer, C. J. *et al.* An Overview of the Main Genetic, Epigenetic and Environmental
Factors Involved in Autism Spectrum Disorder Focusing on Synaptic Activity. *International*
Journal of Molecular Sciences 2020, Vol. 21, Page 8290 **21**, 8290 (2020).

9.   Pocklington, A. J., O'Donovan, M. & Owen, M. J. The synapse in schizophrenia. *European*
Journal of Neuroscience **39**, 1059–1067 (2014).

10.   Lane-Donovan, C. & Herz, J. ApoE, ApoE Receptors, and the Synapse in Alzheimer's
Disease. *Trends in Endocrinology & Metabolism* **28**, 273–284 (2017).

11.   Rizo, J. & Xu, J. The Synaptic Vesicle Release Machinery. *Annu Rev Biophys* **44**, 339–367
(2015).

12.   Rusakov, D. A., Savtchenko, L. P., Zheng, K. & Henley, J. M. Shaping the synaptic signal:
molecular mobility inside and outside the cleft. *Trends Neurosci* **34**, 359–369 (2011).

13.   Luján, R., Shigemoto, R. & López-Bendito, G. Glutamate and GABA receptor signalling in
the developing brain. *Neuroscience* **130**, 567–580 (2005).

14.   Vyklicky, V. *et al.* Structure, function, and pharmacology of NMDA receptor channels.
Physiol Res **63**, (2014).

15.   Diering, G. H. & Huganir, R. L. The AMPA Receptor Code of Synaptic Plasticity. *Neuron* **100**,
314–329 (2018).

16.   Chen, H., Tang, A. H. & Blanpied, T. A. Subsynaptic spatial organization as a regulator of
synaptic strength and plasticity. *Curr Opin Neurobiol* **51**, 147–153 (2018).

17.   Iasevoli, F., Tomasetti, C. & de Bartolomeis, A. Scaffolding proteins of the post-synaptic
density contribute to synaptic plasticity by regulating receptor localization and
distribution: relevance for neuropsychiatric diseases. *Neurochem Res* **38**, 1–22 (2013).

18.   Akin, O. & Zipursky, S. L. Activity regulates brain development in the fly. *Curr Opin Genet*
Dev **65**, 8–13 (2020).

19.   Andreae, L. C. & Burrone, J. The role of neuronal activity and transmitter release on
synapse formation. *Curr Opin Neurobiol* **27**, 47–52 (2014).

20.   Chen, L. F., Zhou, A. S. & West, A. E. Transcribing the connectome: roles for transcription
factors and chromatin regulators in activity-dependent synapse development. *J*
Neurophysiol **118**, 755–770 (2017).

21.   Shen, K. & Scheiffele, P. Genetics and cell biology of building specific synaptic connectivity.
Annu Rev Neurosci **33**, 473–507 (2010).

22.   Sanes, J. R. & Zipursky, S. L. Synaptic Specificity, Recognition Molecules, and Assembly of
Neural Circuits. *Cell* **181**, 536–556 (2020).

23.   Jain, S. & Zipursky, S. L. Temporal control of neuronal wiring. *Semin Cell Dev Biol* (2022)
doi:10.1016/J.SEMCDB.2022.05.012.

24.   Farhy-Tselnicker, I. *et al.* Activity-dependent modulation of synapse-regulating genes in
astrocytes. *Elife* **10**, (2021).

25.   Wilton, D. K., Dissing-Olesen, L. & Stevens, B. Neuron-Glia Signaling in Synapse
Elimination. *Annu Rev Neurosci* **42**, 107–127 (2019).

26.   Chung, W. S., Allen, N. J. & Eroglu, C. Astrocytes Control Synapse Formation, Function, and
Elimination. *Cold Spring Harb Perspect Biol* **7**, (2015).

27.   Bonetto, G., Belin, D. & Káradóttir, R. T. Myelin: A gatekeeper of activity-dependent circuit
plasticity? *Science* **374**, (2021).

28.   Sancho, L., Contreras, M. & Allen, N. J. Glia as sculptors of synaptic plasticity. *Neurosci Res*
167, 17–29 (2021).

29.   Allen, N. J. & Eroglu, C. Cell Biology of Astrocyte-Synapse Interactions. *Neuron* **96**, 697–
708 (2017).

30.   Baldwin, K. T. & Eroglu, C. Molecular mechanisms of astrocyte-induced synaptogenesis.
Curr Opin Neurobiol **45**, 113–120 (2017).

31.   Tan, C. X., Burrus Lane, C. J. & Eroglu, C. Role of astrocytes in synapse formation and
maturation. *Curr Top Dev Biol* **142**, 371–407 (2021).

32.   Watanabe, S. *et al.* Clathrin regenerates synaptic vesicles from endosomes. *Nature 2014*
515:7526 **515**, 228–233 (2014).

33.   Begemann, I. & Galic, M. Correlative light electron microscopy: Connecting synaptic
structure and function. *Frontiers in Synaptic Neuroscience* vol. 8 Preprint at
https://doi.org/10.3389/fnsyn.2016.00028 (2016).

34.   Staffler, B. *et al.* SynEM, automated synapse detection for connectomics. *Elife* **6**, (2017).

35.   Dorkenwald, S. *et al.* Automated synaptic connectivity inference for volume electron
microscopy. *Nature Methods 2017 14:4* **14**, 435–442 (2017).

36.   Chandrasekaran, S. *et al.* Unbiased, High-Throughput Electron Microscopy Analysis of
Experience-Dependent Synaptic Changes in the Neocortex. *Journal of Neuroscience* **35**,
16450–16462 (2015).

37.   Segev, A., Garcia-Oscos, F. & Kourrich, S. Whole-cell Patch-clamp Recordings in Brain
Slices. *J Vis Exp* **2016**, 54024 (2016).

38.   Ippolito, D. M. & Eroglu, C. Quantifying Synapses: an Immunocytochemistry-based Assay
to Quantify Synapse Number. *JoVE (Journal of Visualized Experiments)* e2270 (2010)
doi:10.3791/2270.

39. Maidorn, M., Rizzoli, S. O. & Opazo, F. Tools and limitations to study the molecular
composition of synapses by fluorescence microscopy. *Biochem J* **473**, 3385–3399 (2016).

40. Blanco-Suarez, E., Liu, T. F., Kopelevich, A. & Allen, N. J. Astrocyte-Secreted Chordin-like 1
Drives Synapse Maturation and Limits Plasticity by Increasing Synaptic GluA2 AMPA
Receptors. *Neuron* **100**, 1116-1132.e13 (2018).

41. Stogsdill, J. A. *et al.* Astrocytic neuroligins control astrocyte morphogenesis and
synaptogenesis. *Nature* **551**, 192–197 (2017).

42. Holt, L. M. *et al.* Astrocyte morphogenesis is dependent on BDNF signaling via astrocytic
TrkB.T1. *Elife* **8**, (2019).

43. Nguyen, A. Q. *et al.* Astrocytic Ephrin-B1 Controls Excitatory-Inhibitory Balance in
Developing Hippocampus. *J Neurosci* **40**, 6854–6871 (2020).

44. Glebov, O. O., Cox, S., Humphreys, L. & Burrone, J. Neuronal activity controls transsynaptic
geometry. *Scientific Reports 2016 6:1* **6**, 1–11 (2016).

45. Baldwin, K. T. *et al.* HepaCAM controls astrocyte self-organization and coupling. *Neuron*
(2021) doi:10.1016/j.neuron.2021.05.025.

46. Nguyen, A. Q. *et al.* Astrocytic Ephrin-B1 Controls Excitatory-Inhibitory Balance in
Developing Hippocampus. *J Neurosci* **40**, 6854–6871 (2020).

47. Verstraelen, P., Garcia-Diaz Barriga, G., Larsen, P. H., Timmermans, J.-P. & de Vos, W. H.
Systematic Quantification of Synapses in Primary Neuronal Culture. *iScience* **23**, 101542
(2020).

48. McNamara, G., Difilippantonio, M., Ried, T. & Bieber, F. R. Microscopy and Image Analysis.
Curr Protoc Hum Genet **94**, 4.4.1-4.4.89 (2017).

49. Dani, A., Huang, B., Bergan, J., Dulac, C. & Zhuang, X. Superresolution Imaging of Chemical
Synapses in the Brain. *Neuron* **68**, 843–856 (2010).

50. Risher, W. C. *et al.* Astrocytes refine cortical connectivity at dendritic spines. *Elife* **3**,
(2014).

51. Risher, W. C. *et al.* Thrombospondin receptor α2δ-1 promotes synaptogenesis and
spinogenesis via postsynaptic Rac1. *Journal of Cell Biology* **217**, 3747–3765 (2018).

52. Danielson, E., Lee, S. H. & Fox, M. A. SynPAnal: Software for rapid quantification of the
density and intensity of protein puncta from fluorescence microscopy images of neurons.
PLoS One **9**, (2014).

53. Schmitz, S. K. *et al.* Automated analysis of neuronal morphology, synapse number and
synaptic recruitment. *J Neurosci Methods* **195**, 185–193 (2011).

54. Moreno Manrique, J. F. *et al.* SynapseJ: An Automated, Synapse Identification Macro for
ImageJ. *Front Neural Circuits* **15**, (2021).

55. Dzyubenko, E., Rozenberg, A., Hermann, D. M. & Faissner, A. Colocalization of synapse
marker proteins evaluated by STED-microscopy reveals patterns of neuronal synapse
distribution in vitro. *J Neurosci Methods* **273**, 149–159 (2016).

56. Wang, Y. *et al.* SynQuant: an automatic tool to quantify synapses from microscopy
images. *Bioinformatics* **36**, 1599–1606 (2020).

57. Risher, W. C. *et al.* Thrombospondin receptor α2δ-1 promotes synaptogenesis and
spinogenesis via postsynaptic Rac1. *J Cell Biol* **217**, 3747 LP – 3765 (2018).

58. Irala, D. *et al.* Astrocyte-secreted neurocan controls inhibitory synapse formation and
function. *Neuron* (2024) doi:10.1016/j.neuron.2024.03.007.

59. Berg, S. *et al.* ilastik: interactive machine learning for (bio)image analysis. *Nat Methods* **16**,
1226–1232 (2019).

60. Eisele, R. Calculate the intersection area of two circles.
https://www.xarg.org/2016/07/calculate-the-intersection-area-of-two-circles/ (2016).

61. Irala, D. *et al.* Astrocyte-Secreted Neurocan Controls Inhibitory Synapse Formation and
Function 2. *bioRxiv* (2023) doi:10.1101/2023.04.03.535448.

62. Singh, S. K. *et al.* Astrocytes Assemble Thalamocortical Synapses by Bridging NRX1α and
NL1 via Hevin. *Cell* **164**, 183–196 (2016).

63. Allen, N. J. *et al.* Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via
GluA1 AMPA receptors. *Nature* **486**, 410–414 (2012).

64. Christopherson, K. S. *et al.* Thrombospondins are astrocyte-secreted proteins that
promote CNS synaptogenesis. *Cell* **120**, 421–433 (2005).

65. Kucukdereli, H. *et al.* Control of excitatory CNS astrocyte-secreted protein. 43–45 (2016)
doi:10.1073/pnas.1104977108.

66. Diniz, L. P. *et al.* Astrocyte-induced synaptogenesis is mediated by transforming growth
factor β signaling through modulation of d-serine levels in cerebral cortex neurons.
Journal of Biological Chemistry **287**, 41432–41445 (2012).

67. Eroglu, Ç. *et al.* Gabapentin Receptor α2δ-1 Is a Neuronal Thrombospondin Receptor
Responsible for Excitatory CNS Synaptogenesis. *Cell* **139**, 380–392 (2009).

1126

A)

B)

2) Primary antibody

Excitatory Synapses

Ms-α-Bassoon
GP-α-VGluT1
Rb-α-Homer1

Inhibitory Synapses

Ms-α-Bassoon
GP-α-VGAT
Rb-α-Gephyrin

1) Permeabilization and blocking

3) PBS Wash

4) Secondary antibody

Gt-α-Ms Alexa 488
Gt-α-GP Alexa 647
Gt-α-Rb Alexa 564

Gt-α-Ms Alexa 488
Gt-α-GP Alexa 647
Gt-α-Rb Alexa 564

5) PBS Wash and DAPI

6) Mounting

C) Excitatory Synapses

Homer1   Bassoon   VGluT1   Merged

D) Inhibitory Synapses

Gephyrin   Bassoon   VGAT   Merged

A)

B)

A)

Distance between puncta centers $= d = \sqrt{(y_b - y_a)^2 + (x_b - x_a)^2}$

If $d < r_a + r_b$:

All colocalizations with any overlap are counted
If there is an overlap, the colocalization area is calculated

B)

Distance from x_a to the line of intersection $= x_d = \dfrac{(r_a^2 - r_b^2 + d^2)}{2d}$

Distance from the point of intersection to the midline $= y_d = \sqrt{r_a^2 - x_d{}^2}$

$= Sector_a = r_a^2 \cdot \sin^{-1}\left(\dfrac{y_d}{r_a}\right)$
$= Sector_b = r_b^2 \cdot \sin^{-1}\left(\dfrac{y_d}{r_b}\right)$

$= Triangles = y_d \cdot \left(x_d + \sqrt{x_d{}^2 + r_b^2 + r_a^2}\right)$

$= Area_{coloc} = Sector_a + Sector_b - Triangles$

C)

Red AND Green AND Blue = Triple Colocalization (White)

A Single Cell Suspension

P1 Rat Brain → Cerebral Cortex → Enzymatic digestion → Mechanical digestion → Single cell suspension

B Neuronal Isolation

Negative panning

macrophages

Non-specific binding   microglia   cell debris

Lectin Plate   Anti-Mouse plate   Anti-Rat plate

Positive panning

oligodendrocytes   Astrocytes

Anti-L1   Neurons

C ACM Preparation

Shake off

Astrocytes

ACM concentration by membrane ultrafiltration

ACM

D Dissection/Culture day

AraC   AraC removal   Feeding   **ACM feeding**   **ACM feeding**   Fixation/staining   Imaging

DIV2   DIV3   DIV6   DIV8   DIV11   DIV13

E Bassoon/Homer Excitatory Synapses

control

ACM

Bassoon
Homer1

F

Fold Change in synapse number (colocalization of bassoon and homer1)

| puncta_analyzer | manual_synbot | ilastik | synquant |
| p = 0.009 | p = 0.004 | p = 0.010 | p = 0.015 |

control   ACM   control   ACM   control   ACM   control   ACM

condition

G Bassoon/Gephyrin Inhibitory Synapses

control

ACM

Bassoon
Gephyrin

H

Fold Change in synapse number (colocalization of bassoon and gephyrin)

| puncta_analyzer | manual_synbot | ilastik | synquant |
| p < 0.001 | p < 0.001 | p < 0.001 | p < 0.001 |

control   ACM   control   ACM   control   ACM   control   ACM

condition

A)

B)

	puncta analyzer	manual synbot	ilastik	synquant
P21 α2δ-1 WT				
P21 α2δ-1 KO				

VGlut1
PSD95

C)

Fold change in synapse number (colocalization of VGluT1 and PSD95)

puncta_analyzer — p < 0.001

manual_synbot — p < 0.001

ilastik — p < 0.001

synquant — p < 0.001

condition