

# MODULATION OF SYNAPTIC STRUCTURE AND FUNCTION IN PRIMARY RODENT HIPPOCAMPAL NEURONS BY AUTISM RISK GENES-A HIGH CONTENT IMAGE ANALYSIS

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## Background

- Synaptic dysfunction is a common early event in neuro-degenerative diseases and age-associated cognitive decline.
- Despite a clear link between synaptic spines, synaptic connectivity, neuro-degeneration and neuro-developmental diseases there remains a distinct lack of tools to assay such processes within modern drug discovery work-flows.
- Monitoring synaptic integrity in cultured neurons could serve as readout system for a variety of mental disorders (Figure 1).
- Evotec has developed a drug discovery platform which uses state-of-the-art high content imaging technology for in vitro and ex vivo analysis of synapse number and structure. This platform can be used for target validation studies and also for compound profiling.

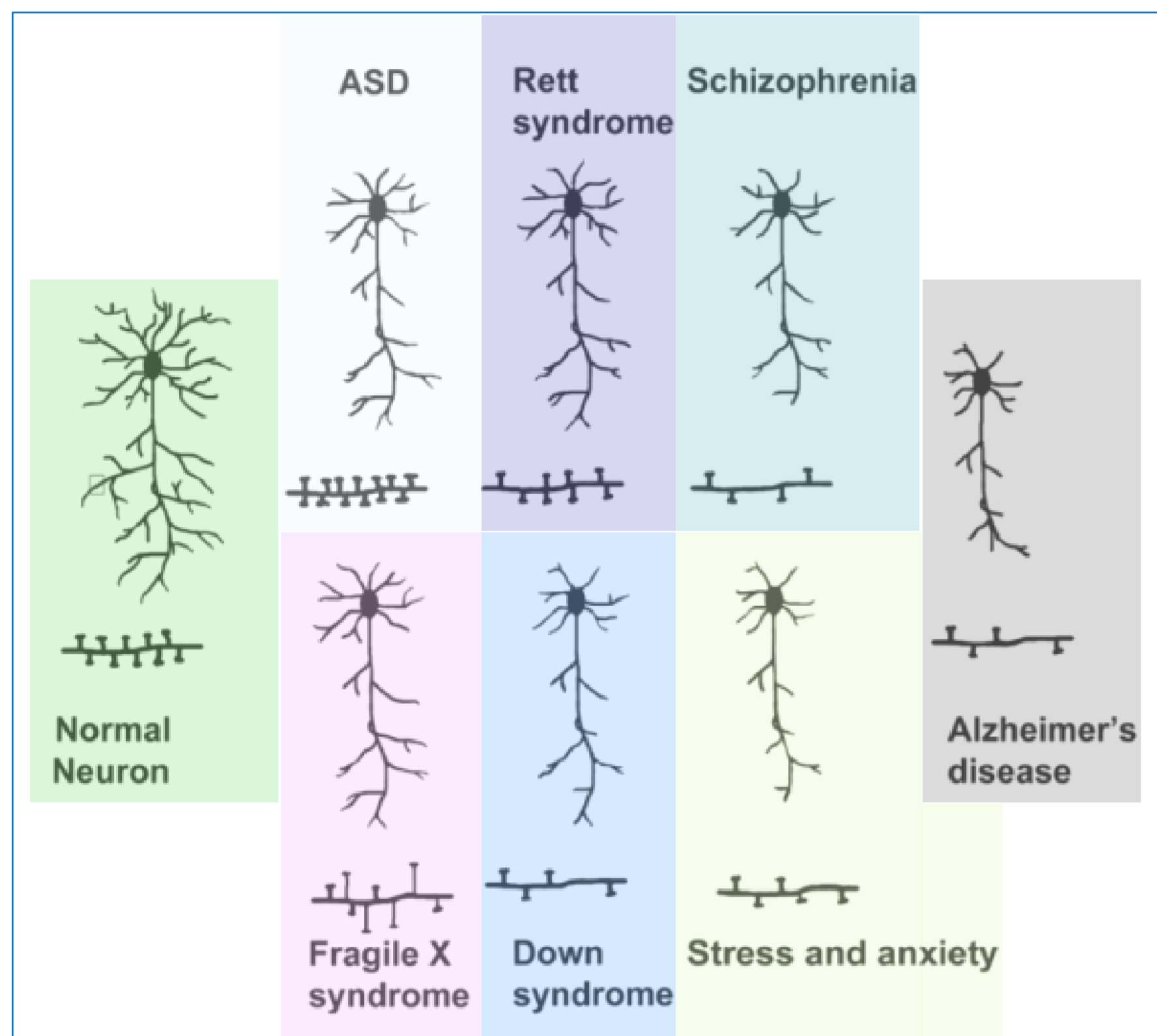


Figure 1: Schematic representation of altered dendritic morphology in neurons associated with specific CNS diseases <sup>1)</sup>

## 1. Automated synapse counting using Evotec developed analysis scripts

Automated image analysis of pre- and post-synaptic markers was performed on primary cultures of hippocampal neurons using in-house developed Acapella™-based scripts (Figure 2A-A''). To determine the sensitivity of the assay, synaptic transmission was pharmacologically enhanced using picrotoxin (PTX) for a period of 3 days. This treatment produced a reduction in the density of synapses (Figure 2C). The effect of PTX could be reversed by inhibiting synaptic connectivity using tetrodotoxin (TTX) and also by selective block of NR2B-subunit-containing NMDA receptors by ifenprodil (Figure 2C).

To model diseases at a synaptic level we have used AAV-mediated shRNAs to knock-down key Autism Spectrum Disease (ASD) related genes <sup>2)</sup>. The resulting "synaptopathy" may be suitable for in vitro phenotypic rescue experiments using small molecule libraries. Preliminary data indicates gene-dependent up- and down-regulation of synapse density (Figure 3).

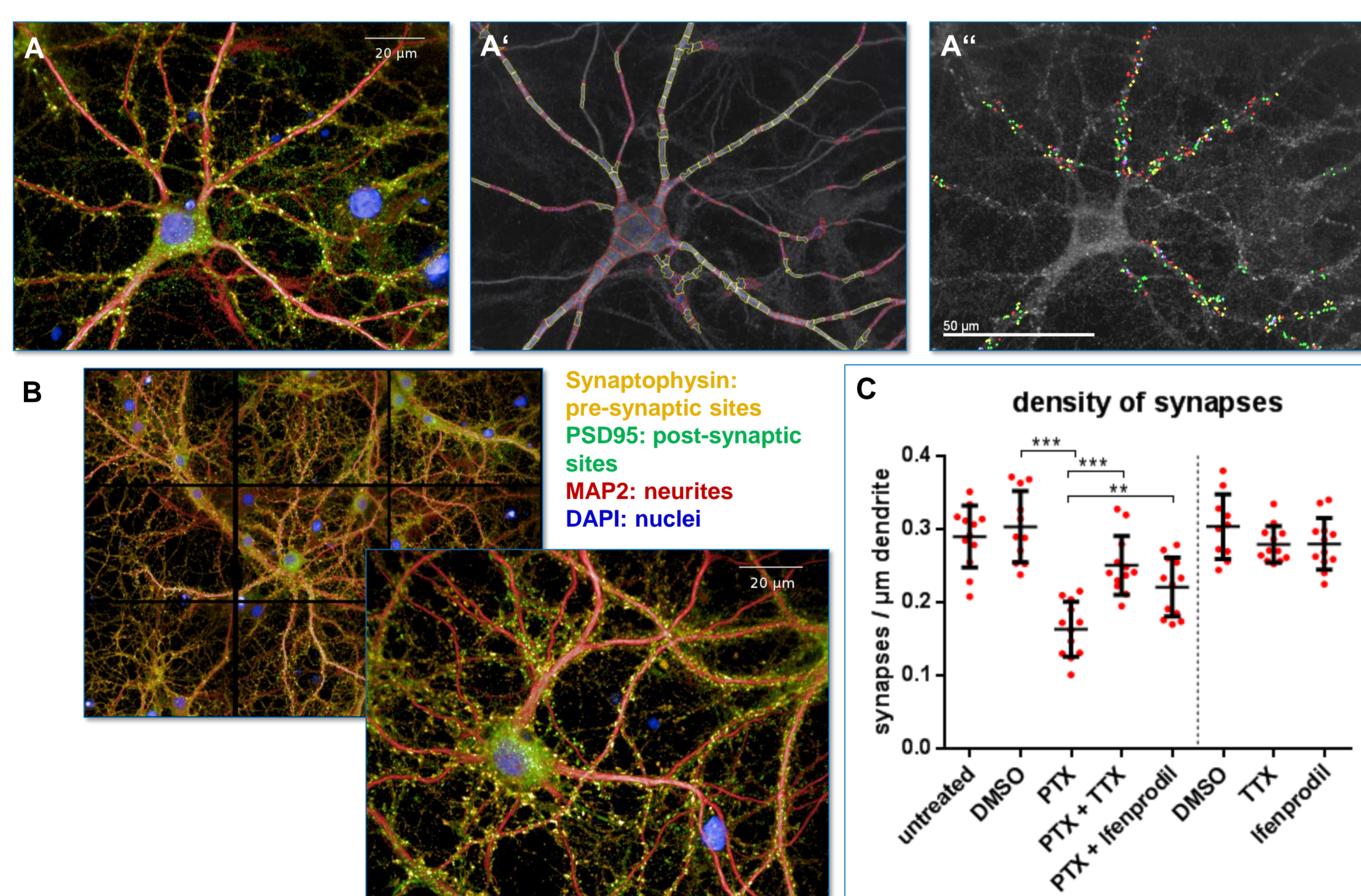


Figure 2: Regulation of synapse number via pharmacological treatment.

Synaptic measurements of rat hippocampal neurons pre-treated with PTX (DIV14-16). Tool compounds were applied at DIV21 for 24h before fixation. Density of synapses were determined by automated image analysis along valid dendrite fragments (yellow in A') using co-localizing signal (A'') of markers for pre-synaptic (B, Synaptophysin, yellow) and post-synaptic sites (B, PSD-95, green) along MAP2 labelled dendrites (B, red) at 60x confocal Opera® images (PerkinElmer Inc.). PTX pre-treatment leads to a reduction of synapse density which can be rescued using 24h TTX or ifenprodil treatment (C).

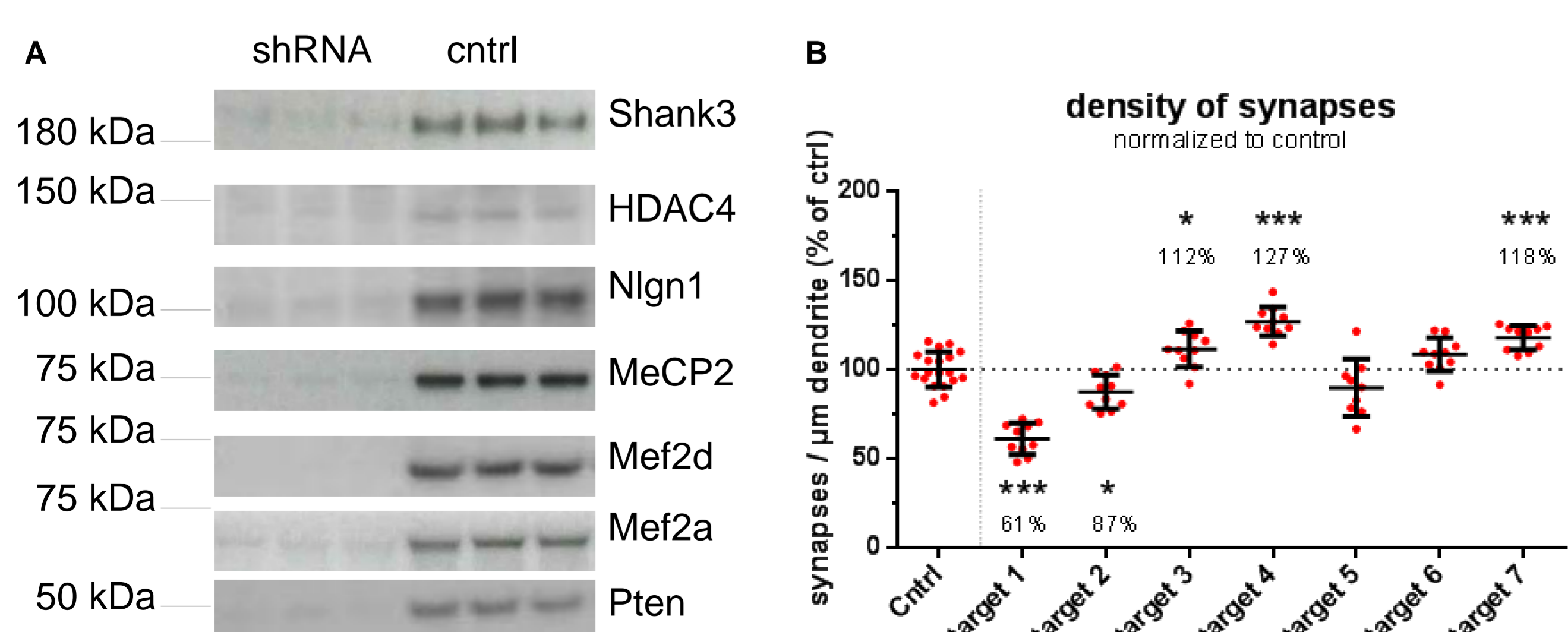


Figure 3: Regulation of synapse number via gene knockdown.

(A) Confirmation of AAV-shRNA mediated knockdown of a selection of Autism spectrum disorder (ASD) linked genes using western blot. (B) Synaptic measurements of rat hippocampal neurons transduced with ASD AAV-shRNAs. Transduction at DIV3 using MOI 1000, fixation at DIV21. Density of synapses was determined using co-localizing signal of markers for pre- and post-synaptic sites along MAP2 labelled dendrites at 60x confocal Opera® images (PerkinElmer Inc.).

## Conclusion

Evotec has developed multiple assays to induce and measure changes in synaptic structure and function in primary cultures of rat and mouse hippocampal neurons.

- Used the Opera® platform and high content image analysis to detect co-localisation of pre- and post-synaptic sites in 96- and 384-well imaging plates
- Developed a reproducible technique for GFP labelling of single neurons in primary cultures to visualize dendritic spines in 3D
- Used electrophysiological measurements to assess how structural or density changes alter functional connectivity (data not shown)
- Modulated risk gene expression by viral transduction in order to derive translational models for ASD
- It is suggested that this platform can be used to identify/validate targets and/or small molecules capable of regulating neuronal synapses. Such information would be beneficial for the development of CNS disease therapies.

## 2. Spine identification using Acapella workflow

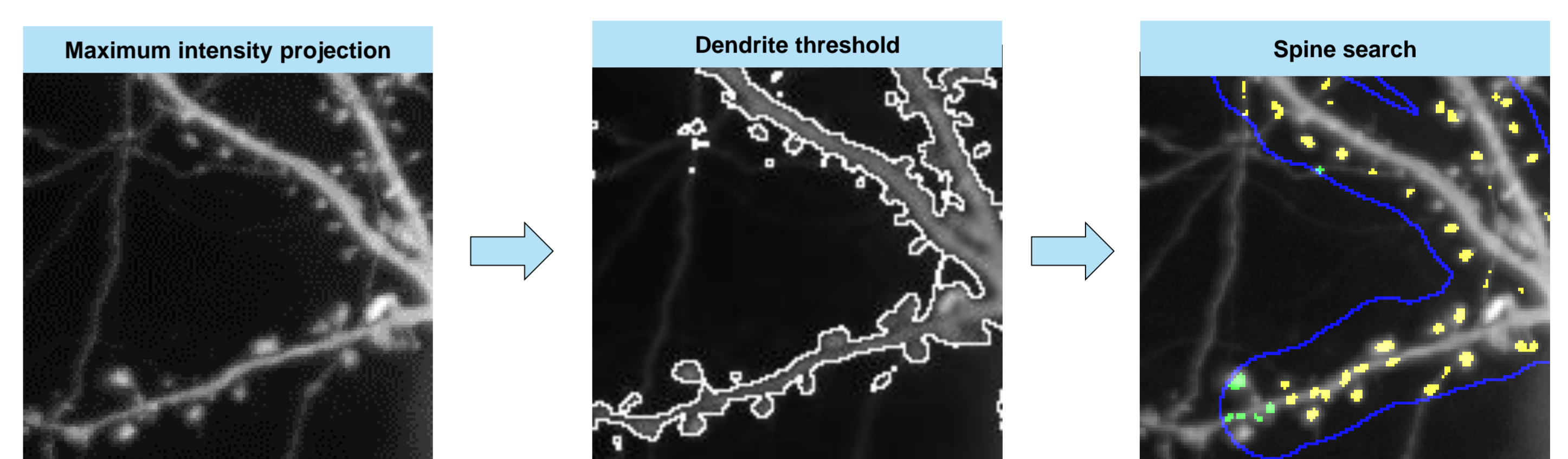


Figure 4: Automated identification of synaptic spines.

Spine identification in rat hippocampal neurons transfected with eGFP. Neurons were transfected at DIV7 using Lipofectamine 2000 (Invitrogen), and fixed at DIV21. Multi-field images were acquired covering the region of interest with up to 154 fields per well using an Opera High-Content Screening System (PerkinElmer Inc.). Analysis was performed using the Columbus image data management and analysis system version 2.4 (PerkinElmer Inc.). Z-series were acquired as 9 planes with 0.5 µm increments. The Acapella script starts with a thresholding step on the maximal intensity projection image to select the dendritic core. Then, 'seed points' for the spine candidates are identified with a texture spot filter method and matched to fit in the spine search area. Finally, the spine length and 3D volume is determined.

Confocal stacks of eGFP-transfected hippocampal neurons were imaged using an Opera® High-Content Screening System. A total of 30 dendritic regions were subjected to analysis using Acapella automated scripts and compared to Imaris Bitplane® workflows.

- Using the current workflows the Imaris method detected more objects as spines. These tended to be more filopodial in shape (Figure 5A-C). These filopodia were defined as pin (Vol<0.25µm<sup>3</sup>, L<0.8µm) or thin (Vol<0.25µm<sup>3</sup>, L>0.8µm) depending on their length.
- Total number of mushroom spines (Vol>0.25µm<sup>3</sup>, L>0.8µm), and stubby spines were approximately the same using the two methods (Figure 5C).
- When tested against a target known to alter spine stability, the Acapella automated detection method was able to detect a significant increase in spine volume after shRNA-mediated target knockdown.

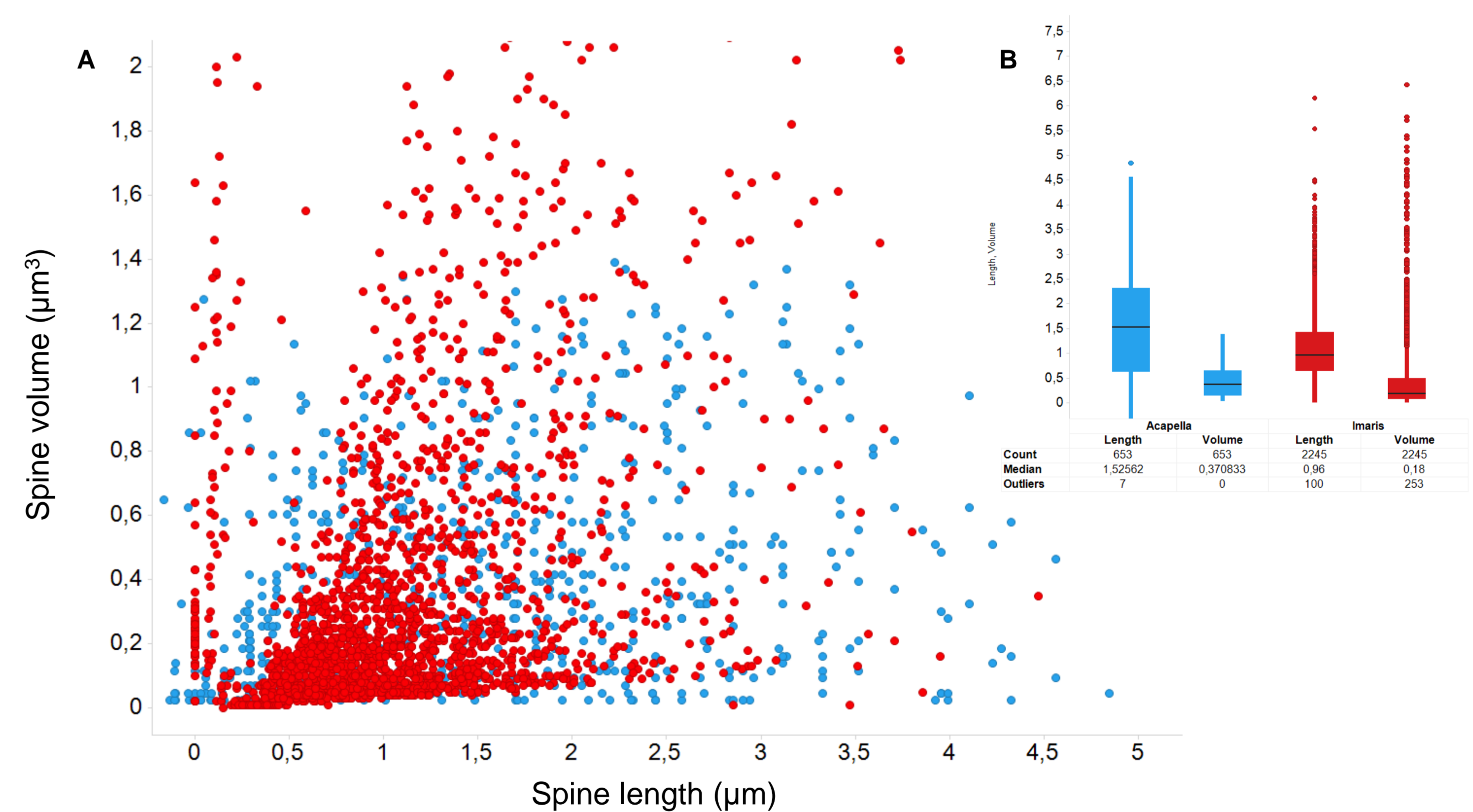


Figure 5: Comparison of spine morphologies.

Data captured and analysed automatically as described in Fig. 4 was compared to semi-manual analysis of the same dataset using Imaris Bitplane software. (A) shows spine volume vs length plotted for each individual spine detected. Here it is clear that Acapella and Imaris detect a slightly different spine population. Based on length and volume as shown in (A) and (B), spines were classified into 4 bins according to spine length and spine head volume <sup>4)</sup>. Imaris detected more pin and thin spines, while the number of stubby and mushroom spines detected was similar (C).

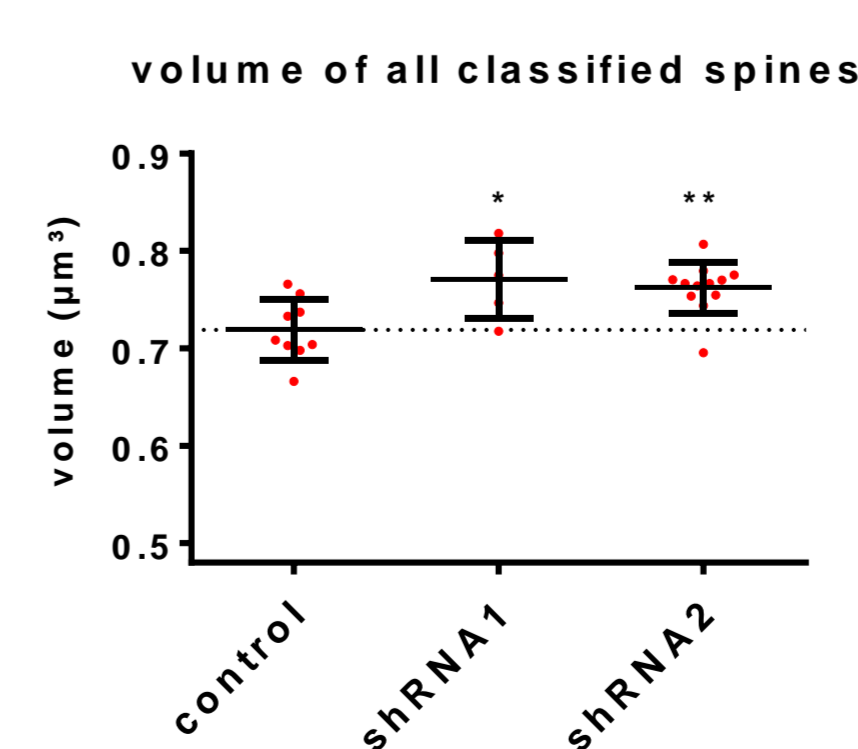


Figure 6: Changes in spine morphology upon genetic knockdown.

Upon the knockdown of a target gene involved in spine stability the Acapella-based automated detection script detected reproducible increases in spine volume. Data is a representative example from 4 independent experiments. One-way ANOVA; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001

## References

- 1) Kulkarni and Firestein (2012) Mol and Cell Neuroscience: 50, 10-20.
- 2) Lanz et al. (2013) Molecular Autism 2013, 4:45.
- 3) Swanger et al. (2011) Molecular Brain 2011, 4:38.
- 4) Koh and Lindquist (2001) BIOS 2001, 48-59.