A HIGH CONTENT *IN VITRO* SCREEN FOR MEASURING REGULATION OF SYNAPSES BY GENETIC AND PHARMACOLOGICAL TARGET MODULATION

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Introduction

- Synaptic dysfunction is a common early event in neuro-degenerative diseases and age-associated cognitive decline
- Monitoring synaptic integrity in cultured neurons could serve as readout system for a variety of mental disorders (Figure 1).
- Importantly, loss of synaptic connectivity may prove to be a reversible phenomenon suitable for drug intervention.
  In contrast, neuronal loss occurring at disease end-stage is unlikely to be readily reversible. Indeed current therapeutics for Alzheimer's disease, the most prevalent cognitive disorder in the world today, focus on enhancing the signaling properties of the remaining neuronal population rather than addressing the disease progression itself.
- Despite a clear link between spines, synaptic connectivity, neuro-degenerative and neuro-developmental diseases there remains a distinct lack of tools to assay such processes within modern drug discovery work-flows.

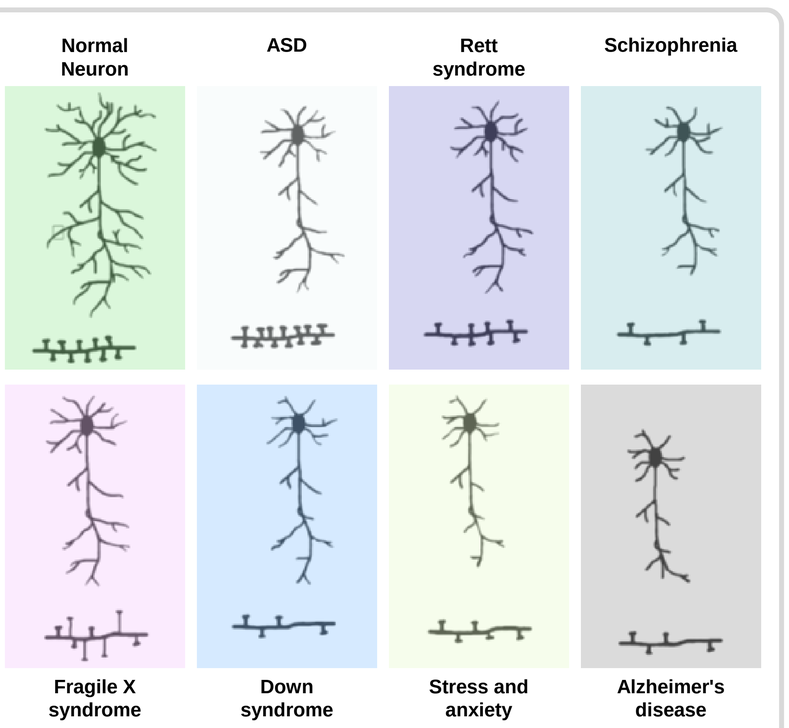

Figure 1: Schematic representation of altered dendritic morphology in neurons associated with specific CNS diseases[1)]

- Evotec has developed a drug discovery platform which uses state-of-the-art high content imaging technology which can be used for *in vitro* and *in vivo* analysis of synapse number and structure. This platform can be used for target validation studies and also for compound profiling.
- This builds on about 20 years of CNS and drug discovery experiences at Evotec and combines Opera® based imaging with sophisticated analysis tools.

1. A comprehensive set of *in vitro* and *in vivo* tools

The Evotec platform studies CNS diseases by modulating risk gene expression using Adeno-Associated Virus (AAV) mediated gene transfer or knock-down. On the one hand an *in vitro* neuronal culture system can be used for phenotypic assays or compound screening. On the other hand *in vivo* interference with specific signaling path-ways can be tested using AAVs targeted to specific brain regions via stereotactic injections. Both of these approaches use high content analysis to measure effects at the synaptic level. This enables identi-fication and validation of disease relevant targets and in the case of *in vitro* models provides a platform for small molecule screening.

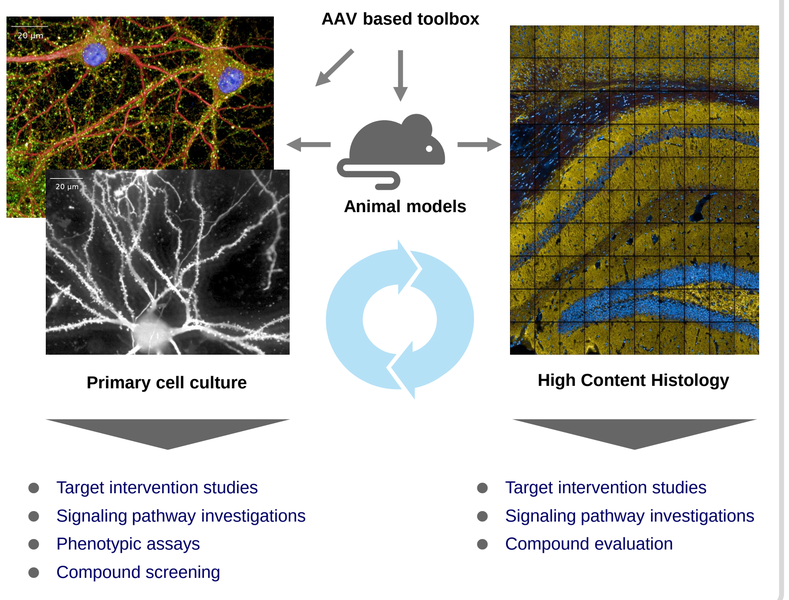

Figure 2: Overview of target and compound validation workflow

2. Automated synapse detection

The assay we have developed measures the co-localization of pre- and post-synaptic markers and is used to quantify the density of synapses. It can also be used to measure the effect of genetic or pharmacological modulation of targets at the synaptic level.

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 3B-B"). 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 3C). The effect of PTX could be reversed by inhibiting synaptic connectivity using tetrodotoxin (TTX) and also by a selective block of NR2B-subunit-containing NMDA receptors by ifenprodil (Figure 3C).

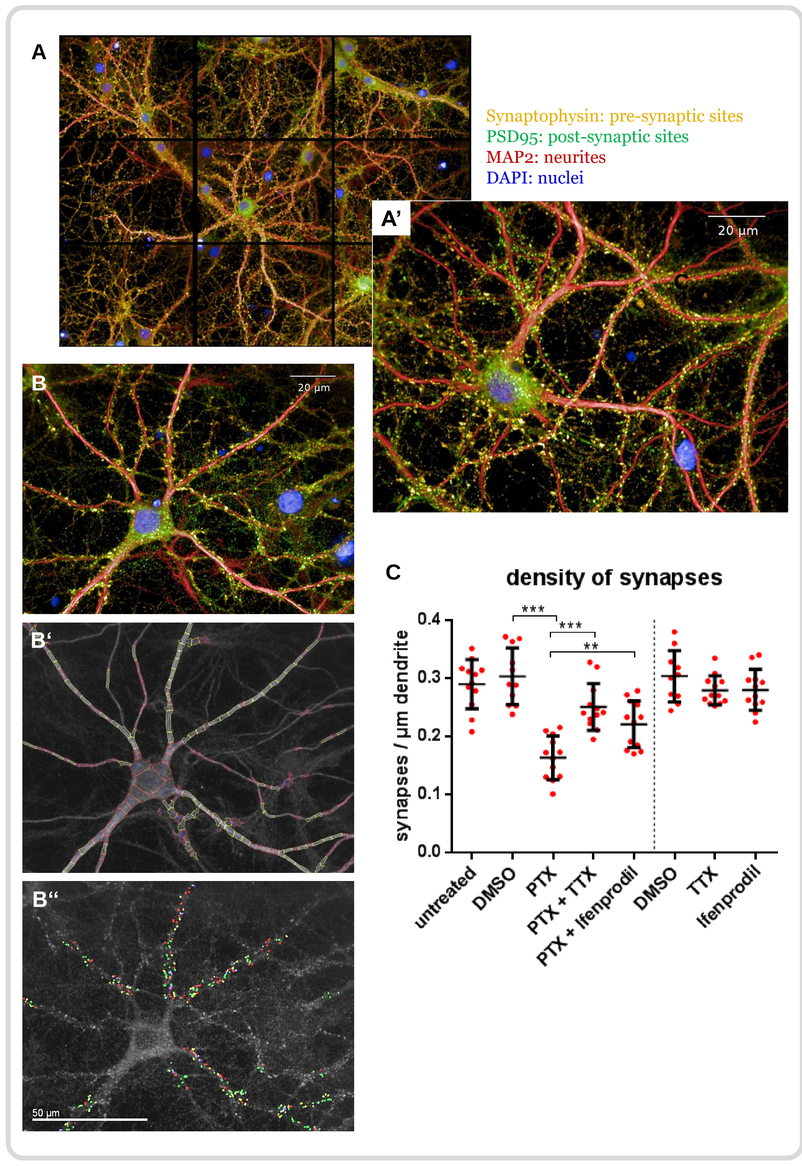

Figure 3: Regulation of synapse number via pharmacological treatment. Synaptic measurements of rat hippocampal neurons pre-treated with PTX (DIV14-16). Tool compounds were applied at DIV21 24h before fixation. Density of synapses was determined by automated image analysis along valid dendrite fragments (yellow in B') using co-localizing signal (B") of markers for pre- (A, Synaptophysin, yellow) and post-synaptic sites (A, PSD-95, green) along MAP2 labelled dendrites (A, red) at 60x confocal Opera® images. PTX pre-treatment leads to a reduction of synapse density which can be rescued using 24h TTX or ifenprodil treatment (C).

3. Spine identification using Evotec developed analysis scripts and Acapella workflow

To complement the synapse density measurements described above, we also developed a reproducible technique for visualization of dendritic spine size and morphology by eGFP or RFP transfection (Figure 4). Individual neurons within each culture are labelled and are suitable for automated 3D high content measurements of spine morphology using Evotec developed Acapella scripts (Figure 5). This method is suitable for pharmacological intervention protocols but also enables to analyze the direct effects of AAV-mediated gene over-expression or knock-down on spine density, size and morphology.

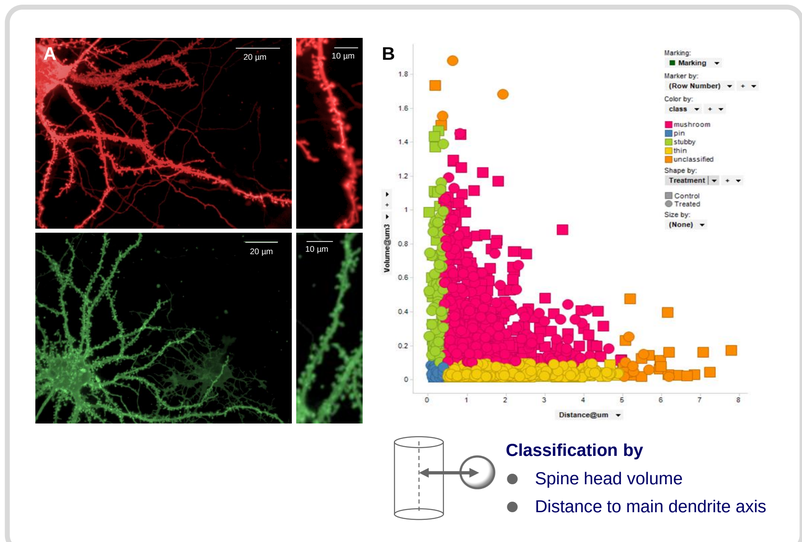

Figure 4: (A) Mature primary rat hippocampal neurons transfected with RFP (red) or eGFP (green) to visualize dendritic spines for quantification and classification into subtypes (B). Based on length and volume, spines were classified into 4 bins according to spine length and spine head volume[3)].

Conclusion

Evotec have 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-localization of pre- and postsynaptic 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 live Ca^{2+} imaging at the FLIPR to assess the neuronal network activity of the culture (data not shown)
- Modulated risk gene expression by viral transduction to derive translational models for ASD
- This platform can be used to identify/validate targets and/or small molecules capable of regulating neuronal synapses. Such information will be integral for the development of CNS disease therapies.

4. *In vitro* modeling of synaptopathies for phenotypic screening

Disruption of synaptic structure and function is thought to be a major determinant of neuro-degenerative and psychiatric disorders. One example is Autism spectrum disorder (ASD); primarily characterized by behavioral and social impairments. Numerous genes have been linked to this disorder, many of which are associated with the post-synaptic density. To model these diseases at a synaptic level Evotec has used AAV-mediated shRNAs to knock-down key ASD related genes[2)]. 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 6).

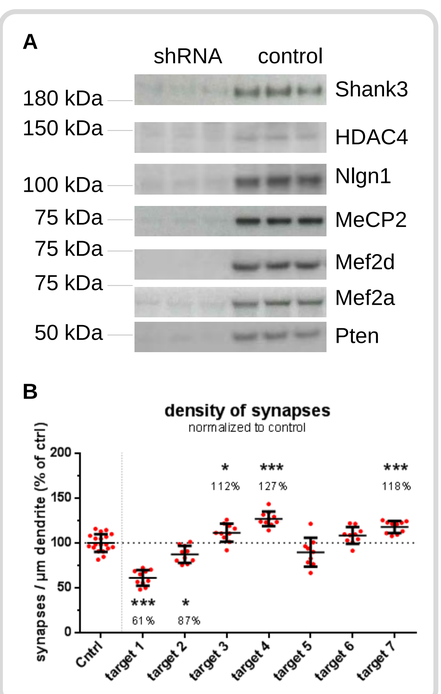

Figure 6: (A) Confirmation of AAV-shRNA mediated knockdown of a selection of Autism spectrum disorder (ASD) linked genes using western blot. (B) Synaptic measurements of rat hippocampal neurons transduced with ASD AAV-shRNAs. Transduction at DIV3 using MOI 1000, fixation at DIV21. Density of synapses was determined using co-localizing signal of markers for pre- and post-synaptic sites along MAP2 labelled dendrites at 60x confocal Opera® images. For this transduced cells only were analyzed.

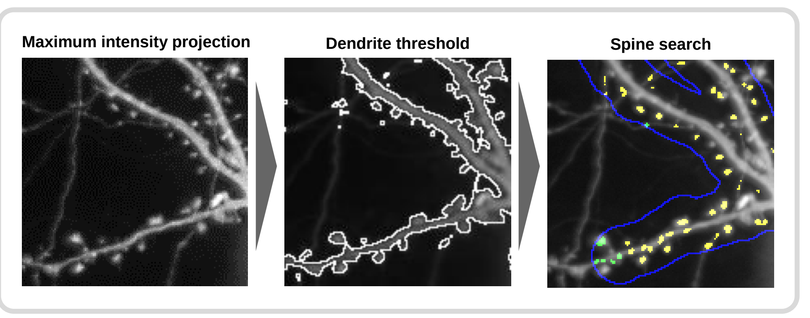

Figure 5: Automated identification of synaptic spines.
Spine identification in rat hippocampal neurons transfected with eGFP. Neurons were transfected at DIV7 using Lipofectamine 2000 (Invitrogen), and fixed at DIV21. Multi-field images were acquired covering the region of interest with up to 154 fields 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.

References

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

Statistical analysis was performed by ONE-way ANOVA and Dunnet's multiple comparison;
* p<0.05; ** p<0.01, *** p<0.001; data are displayed as dot plots with mean ± SD