Functional biomarker of rare and familiar diseases: NPC1 knockout neuronal networks phenotyped with HTS micro electrode arrays and artificial intelligence machine learning methods

Xiao Feng[1], Monica Segura-Castell[2], Luise Schultz[2], Konstantin Jügelt[2], Olaf H.-U. Schröder[2] Jiankai Luo[1], Benjamin M. Bader[2]

1 University Medicine Rostock, Albrecht-Kossel-Institut for Neuroregeneration, Rostock, Germany; 2 NeuroProof GmbH, Rostock, Germany.

Introduction

Background: There is an urgent need to develop fast and reliable disease models in rare diseases which can be used for screening in a standardized manner. One strategy is the development of more predictive pre-clinical in vitro models for gene-associated diseases such as Niemann-Pick Disease Typ C1. Niemann-Pick Type C disease (NPC) is an autosomal recessive neurodegenerative disease caused by a mutation in either the NPC1 gene (in 95% of cases) or the NPC2 gene (in 5%). The pathological changes in NPC1 are characterized by the excessive storage of unesterified cholesterol in lysosomes. NPC Patients show increasing loss of motor control, seizures and other neuropathological Symptoms.

Objectives: Using cell cultures from NPC1 knockout mice, we aimed to identify a correlation between the disease-associated genotype and its functional *in vitro* phenotype.

Summary and Conclusion

We demonstrate that NPC1 knockout cell culture exhibit a significantly different functional phenotype shown by different activity levels and neuronal communication. Moreover, the functional development into mature neuronal networks is affected by the knockout of the NPC1 gene. This specific phenotype can partially rescued by compound addition.

In conclusion, we present a means to functionally phenotype gene-associated disease *in vitro* models by multi-variate and machine learning methods and thus provide functional biomarkers for screening and rank small molecules or natural compounds based on their resuce efficacy of disease genotype-associated functional phenotypes towards isogenic controls or wild type phenotypes.

Methods

Primary culture: P1 mice were genotyped and *npc1* knockout and homozygous litters selected for cortical pyramidal neurons isolation at P2 according to the protocol by Brewer & Torricelli 2007[(1)]. **Genotyping:** PCR with primer sets for knockout: 5'-GGTGCTGGACAGCCAAGTA-3' and 5'-TGAGCCCAAGCATAACTT-3' and for wildtype: 5'-TCTCACAGCCACAAGCTTCC-3' and 5'-CTGTAGCTCATCTGCCATCG-3'. **MEA culture:** 48-well MEAs were coated with PEI/Laminin. Recording was performed with Maestro MEA System (Axion BioSystems). **Immunocytochemistry:** cells were PFA-fixed, permeabilized and incubated overnight with mouse anti-ßIII tublin, 1:1000 (Santa Cruz) and GFAP, 1:500 (Agilent) and goat anti-mouse Alexa 488, goat anti-Rabbit Cy3, 1:500. Filipin 0.5 mg/ml (Polysciences). **Data analysis:** multi-parametric data analysis of 204 spike train parameters was performed using NPWaveX Software (NeuroProof). “Effect Score”: Z’ factor-based projection of multiple parameters into a single layer parameter.

Results

Structural/morphological effects of NPC1 knockout

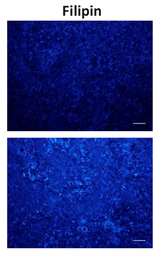


Figure 1. TOP: Cultivation of WT and NPC1-/-mouse cortical pyramidal neuron in 48-well MEA plate with 16 electrodes per well. .Right: wild type (WT) and NPC1-/- cortical pyramidal neuron were labeled by Filipin (blue) at DIV28. Scale bar, 50um.

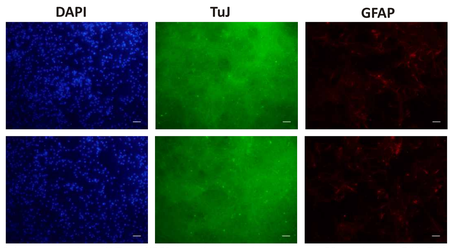


Figure 2: Immunostaining of WT and NPC1-/- cortical pyramidal neurons at 28 days in vitro (DIV). Tuj1 neurons (green), GFAP Glial cells (red), DAPI for nuclei (blue). Scale bar, 50um. Except for the differences in filipin staining (Figure 1), no general morphological differences are observed.

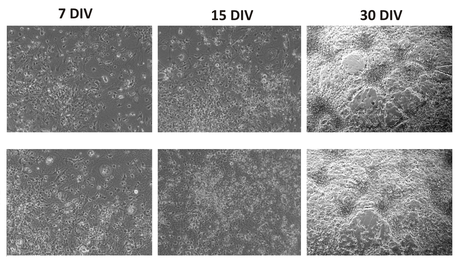


Figure 3: Phase contrast images of neuronal networks between 7 and 30 DIV. Cells growing under same conditions as in 48 well MEAs (coating, density). No general morphological differences are observed.

NPC1 knockout affects functional phenotype

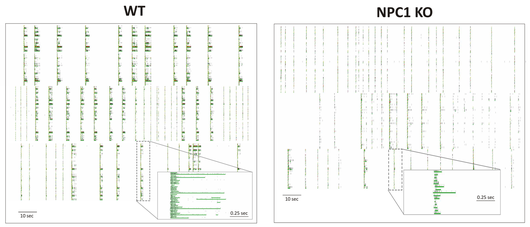


Figure 4: Three example Spike trains for NPC1 knockout and wild type at 21 DIV show a strong phenotypic difference in activity patterns. Spiking and bursting is reduced accompanied by weaker bursts (insert image). Green bars represent detected bursts. Two unit separation was performed.

NPC1 knockout delays functional development

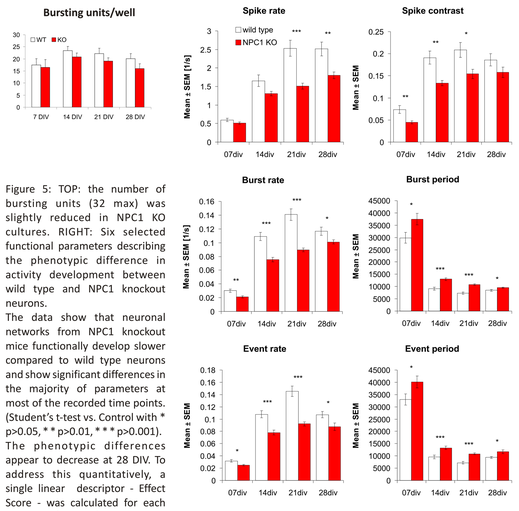


Figure 5: TOP: the number of bursting units (32 max) was slightly reduced in NPC1 KO cultures. RIGHT: Six selected functional parameters describing the phenotypic difference in activity development between wild type and NPC1 knockout neurons.

The data show that neuronal networks from NPC1 knockout mice functionally develop slower compared to wild type neurons and show significant differences in the majority of parameters at most of the recorded time points. (Student’s t-test vs. Control with * p>0.05, * * p>0.01, * * * p>0.001).

The phenotypic differences appear to decrease at 28 DIV. To address this quantitatively, a single linear descriptor - Effect Score - was calculated for each time point (Figure 7).

Compound-mediated rescue of NPC1 knockout phenotype

Multi-parametric data complicate the ranking for identification of optimal assay time points. Our NeuroProof Effect Score allows ranking of phenotypic differences which are multi-parametrically described based on a single readout: a generic analysis of multiple parameters out of the 204 parameters selected by an optimization algorithm, which enables an objective, clear and easy-to-compare documentation of the phenotypic relationship of the complete phenotypic data.

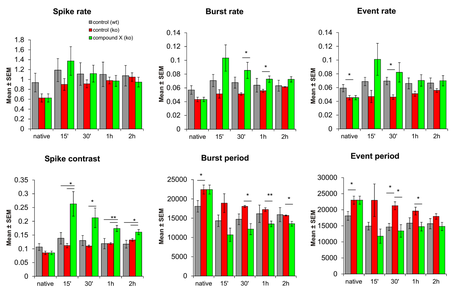


Figure 6: Functional rescue of NPC1 KO induced phenotype shown in six example parameters. Absolute activity values are changed towards control wild type levels after acute addition of a compound (compound X). The rescue effects are stronger within the first hour after compound addition followed by a reduction of rescue efficacy. DIV29, $n_{control}$=5, $n_{NPC1\text{-}KO}$=3, $n_{NPC1\text{-}KO+c1}$=5. Student’s t-test vs. Control with * p>0.05, * * p>0.01, * * * p>0.001.

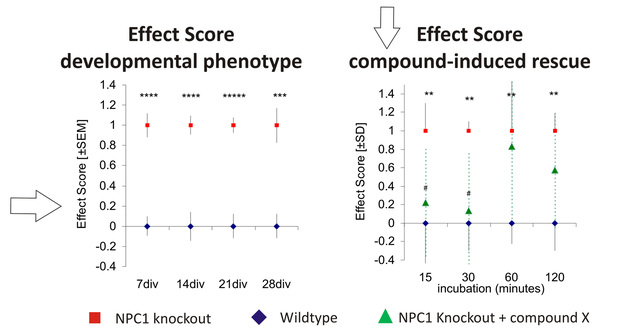
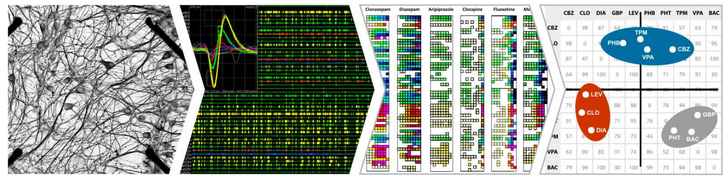


Figure 7: “Effect Score” calculation: Projection of up to 204 parameters into a single parameter allows projecting the complete functional finger print into one dimension and thus, LEFT: identifying the optimal time point showing the highest phenotypic difference (here 21 DIV). Wild type is set to “0”, NPC1 knockout is set to “1”, $n_{control}$=14|14|15|12, $n_{NPC1\text{-}KO}$=12|17|15|12. RIGHT: the evaluation of test compounds which rescue the disease-related phenotype towards wild-type conditions. Wild type is set to “0”. NPC1 knockout is set to “1”. Acute addition of compound X results in a partial rescue of KO-induced functional phenotype towards wild type conditions. Number of experiment: $n_{control}$=5, $n_{NPC1\text{-}KO}$=3, $n_{NPC1\text{-}KO+c1}$=5. Student’s t-test with vs. Control, # vs. NPC1 KO with p>0.05, p>0.01, p>0.001, p>0.0001, p>0.00001 (number of *|#)). The calculated Z-factor (describing the effect size) is optimized to find the best discrimination between WT and NPC1 KO. The “effect score” describes the rescue. For more information on z’-factor, see Kuemmel *et al.* 2010 [(2)] and Kozak *et al.* 2010 [(3)].

NeuroProof Technology

Phenotypic Screening with MEA-Neurochips

Neuronal Cell Culture | **Phenotypic Multichannel Recording** | **Multiparametric Data Analysis** | **Pattern Recognition**



Primary murine cell culture:
- Frontal Cortex
- Hippocampus
- Midbrain
- Spinal Cord/DRG

Divers human iPSC s neurons

Network spike trains and single neuron action potential

Over 200 descriptors at baseline and compound treatment
- General activity
- Synchronization
- Oscillation
- Burst structure

Data base with functional fingerprints of over 100 basic and clinically compounds
Murine brainspecific reference activit patterns for human iPSC neuron

Disease related brain tissue culture Acute and chronic compound induced disease rescue models

High resolution of spatio-temporal network activity patterns

Specific in vitro assays with optimized compound or disease related parameter selection

Similarity and differentiation from known compound effects and comparison of iPSC-neural phenotypes

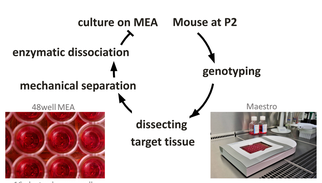
MAESTRO Recording System

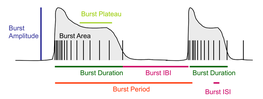
Electrophysiological multiwell multichannel electrode array system for extracellular recording of cortical network activity. From left to right: Maestro recording system (Axion BioSystems, Inc., Atlanta); 12- and 48-well MEAs; electrode area of 12-well MEA with cortical culture at 12 days in vitro; spike activity heat map of an 12-well MEA with 64 electrodes per well

Multiparametric Characterization of Neuronal Network Activity

Read out:
- Extracellular action potentials on a single neuron and network activity level
- Spatio-temporal activity changes as well as synchronicity and oscillation in time scales of spikes and bursts

Each specific spike train is described by 200 parameters in 4 categories:



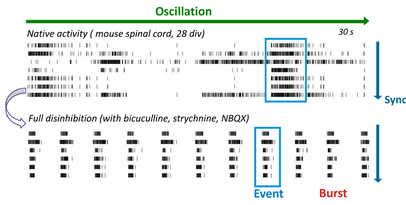


1 General Activity
e.g. spike rate, burst rate, burst period, percent of spikes in burst

2 Burst Structure
e.g. number, frequency and ISI of spikes in bursts; burst duration, amplitude, area, plateau position, plateau duration

3 Oscillation
Variation over time as an indicator for the strength of the oscillation; in addition e.g. Gabor function parameters fitted to autocorrelograms

4 Synchronization
Variation within the network as an indicator for the strength of the synchronization; in addition e.g. simplex synchronization, percent of units in synchronized burst



1 - Nat Prot. (2)6, pp1490-1498
2 - Biomol Screen 15(1):95-101
3 - RNA Biol.7(5):615-20.