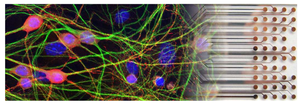

Enhanced predictive drug development: Functional screening of the GABA$_A$R 1 subunit using receptor-specific RNAi, MEA recordings and substance classification

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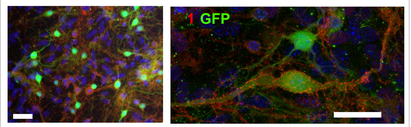
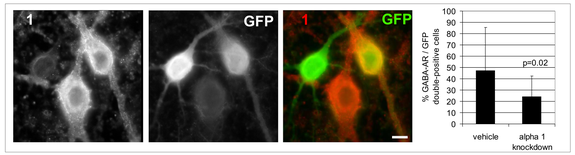
Introduction

The GABAA receptor is involved in the etiology of epilepsy, anxiety and post-traumatic stress disorders (PTSD) and is a well known target for new anxiolytics with high potential. The use of more predictive cell-based in vitro approaches potentially reduces the failure rate in drug development of new compounds. Neuronal networks of primary embryonic dissociated cell cultures grown on microelectrode arrays (MEAs) offer a highly predictive pre-clinical test approach to characterize the potential of novel compounds. The changes in the activity pattern in response to chemical stimulation are reproducible and substance-specific, and thereby applicable as a read-out system in cell-based drug screening. In combination with RNAi-mediated knockdown of specific receptor subunits this approach allows a very specific functional analysis of compounds. We infected frontal cortex cultures grown on MEA neurochips with lenti-viruses containing two specific miRNAs against the mRNA of the GABA$_A$R 1 subunit and with vehicle controls. We evaluated the knockdown effects by fluorescence microscopic and Westernblot quantification and functionally chararacterized the response to the specific 1 agonist Zolpidem by MEA-recording and similarity analyses.

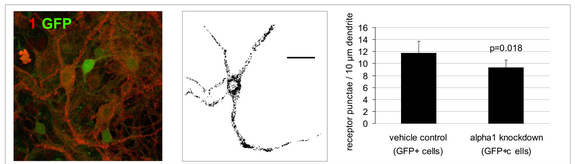
Results



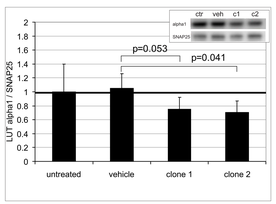
1 GABA$_A$R 1-knock-down in primary neuronal networks transduced with miRNAs against GABA$_A$R 1 subunit including GFP (green), GABA$_A$R 1 co-stained (red), nuclei (blue); bars=30 µm



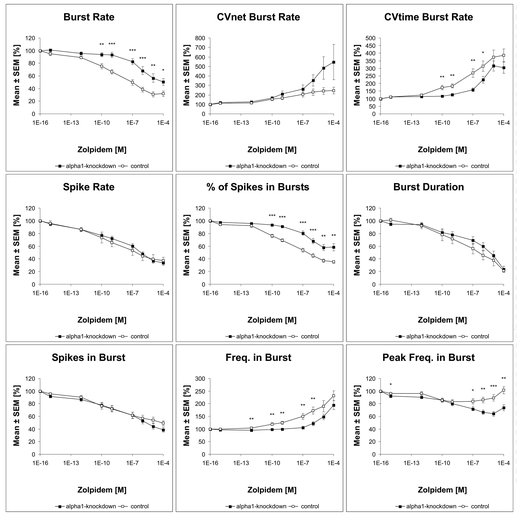
2 Cell type-dependent 1-knockdown: GABA$_A$R 1 immuno-staining revealed that GFP+ cells (1-knockdown) sometimes still contain 1-subunits. A major observation was that strong GFP+ cells exhibit less 1 and vice versa. The ratio of GFP+/ 1+ cells decreased significantly by ~50 % indicating that low 1 abundance was knocked down below the threshold for counting as a 1 cell. Counting was performed manually at a fluorescence microscope, bar=10 µm; n=4x10 fields.



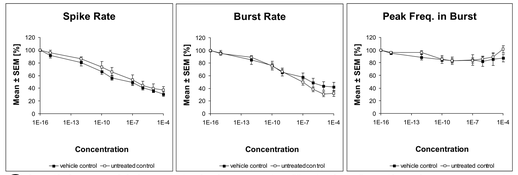
3 Reduction of dendritic GABA$_A$R punctae: semi-automatic quantification of receptor punctae revealed a 25 % reduction after 1-knockdown. The quantification was performed with the ImagaJ particle count plugin after image erosion and subsequent dilatation on a binary image (center). The average number of receptors in GFP+ vehicle control cells was equal to untreated GFP-negative cells (data not shown); n=4.



4 Semi-quantitative Western-analysis of 1-knockdown in whole cell lysates showed an overall knockdown efficacy of approx. 25 %. However, as shown in Fig. 2 only 50 % of all GFP+ neurons contain 1-GABA$_A$Rs. Furthermore, the two tested siRNA clones are driven by the CaMKII promotor and, therefore, only excitatory neurons are effected by the knockdown. Hence, it has to be taken into account that the apparently weak knockdown efficacy corresponds to a relatively small cell population of GABA$_A$R containing neurons; n=8.



5 Functional characterization of 1-knockdown revealed altered burst structure: the response to the GABA$_A$R 1-agonist Zolpidem changed dramatically after knockdown of 1 subunits. A significant right-shift of the curve is observed for burst rate and % spikes in burst. Interestingly, spike rate, spikes in burst and burst duration are not changed. Together, this indicates an altered burst structure due to lack of received inhibitory inputs: despite a decreasing spike rate, more of the single spikes are focussed in bursts as indicated by more bursts and a higher % of spikes in bursts. Yet, only the frequency in bursts is increased but neither the burst duration nor the absolute number of spikes in burst. These data suggest that the composition of subunits in GABA$_A$Rs influences the overall network activity in terms of burst behaviour.



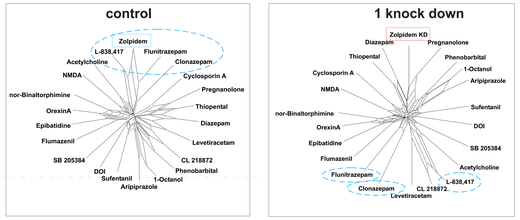
6 Lentivirus vehicle control does not effect the functional response to Zolpidem: the comparison between untreated and vehicle control cells showed that the viral infection with vehicle controls did not effect the connectivity significantly.

Zolpidem	n	7	10	10	10	10	10	10	10	97
Reference	MoId	1,E-15	1,E-12	1,E-10	1,E-09	1,E-08	1,E-06	1,E-05	1,E-04	#
Flunitrazepam	FLN	16	13	39	31	49	67	78	85	97
Levetiracetam	LEV	1	15	54	56	57	43	47	42	44
L-838,417	L838417	0	21	50	71	52	59	45	18	44
SB 205384	SB2	14	29	40	35	40	39	30	45	35
nor-Binaltorphimine	BNP	3	16	33	34	43	24	32	32	29
Clonazepam	CLO	0	1	29	29	43	35	42	34	27
CL 218872	CL2	0	29	34	44	20	30	16	2	24
Acetylcholine	ACH	3	7	16	17	22	35	21	13	18
Flumazenil	FLM	3	22	29	15	13	4	8	13	14
Olanzapine	OZP	34	6	0	1	6	20	34	40	14

Zolpidem knock down	n	11	12	13	13	13	13	13	11	99
Reference	MoId	1,E-15	1,E-12	1,E-10	1,E-09	1,E-07	1,E-06	1,E-05	1,E-04	#
Diazepam	DIA	2	6	18	23	25	30	39	57	25
Levetiracetam	LEV	4	9	18	23	31	39	38	26	24
Thiopental	THP	20	24	28	29	27	18	25	17	24
Flumazenil	FLM	11	21	23	16	25	21	22	17	20
Epibatidine	EPI	18	37	25	32	16	12	9	3	19
Pregnanolone	PRE	0	14	24	28	22	18	15	23	18
Clonazepam	CLO	1	2	4	6	23	35	34	30	17
Cyclosporin A	CSA	7	7	12	14	18	22	11	25	14
Tacrolimus	TAC	5	16	19	25	16	15	11	4	14
nor-Binaltorphimine	BNP	1	18	12	10	12	22	22	11	14

Subst	ZOL	ZOL-RNAi
Reference n	97	99
Flunitrazepam	97	13
Levetiracetam	44	24
L-838,417	44	12
SB 205384	35	20
nor-Binaltorphimine	29	25
Clonazepam	27	8
CL 218872	24	18
Acetylcholine	18	24
Flumazenil	14	17
Olanzapine	14	14
AMPA	14	11
Diazepam	13	7
Oxotremorine	13	19
OrexinA	12	4
DOI	11	14
Epibatidine	10	12
Memantine	10	9
Tramadol	9	10
XII 093	9	2
Propofol	9	4
Carbamazepine	8	9
Wortmannin	8	14
Sufentanil	8	3
NMDA	8	9
Phenobarbital	8	12

7 Similarity to GABA$_A$ receptor-related modulators decreased by knock down: Shown are the TOP10 results of >100 substances in the NeuroProof database. The classification analysis revealed a high similarity between the response profiles of Zolpidem and GABA$_A$ receptor-stimulating substances such as Fluniztrazepam, Levetiracetam, L838.417, SB205.384, Clonazepam, CL218.872 and Flumazenil. This result is significantly altered after 1-knockdown: similarities to drugs such as the AChR modulator Epibatidine, or the immunosupressants Cyclosporine A and Tacrolimus. More importantly, the mean similarity values (green column) decreased dramatically in, indicating a higher degree of noise.



8 Phyllogenetic network representation of the classification results: Zolpidem exhibits a high similarity to L-838.417 and Flunitrazepam indicated by short tree branches forming a cluster (dotted circle, left). After 1-knockdown this cluster is diminished and previously highly similar substances are even located at the opposite side of the tree (dotted circles, right). Furthermore, the branching points are closer to the center, indicating a higher degree of unsimilarity.

Summary

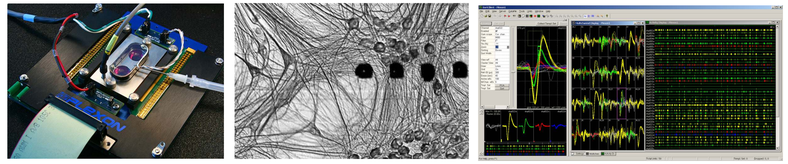
1. **Sucessfull knockdown as indicated by:**
   fewer 1-subunits in lysates, fewer GABA$_A$R-positive cells, fewer dendritic receptor punctae
2. **Sucessfull functional knockdown as indicated by:**
   very significant right-shift in the concentration- response curve
3. **Spike train analysis revealed:**
   strongest functional differences in burst structure but no effects on spike rate
4. **Substance classification against NP database showed:**
   smaller similarity to GABA$_A$R-related compounds after RNAi

Together, these data indicate a high specificity of this combined RNAi and functional MEA-recording approach. Furthermore, it provides a means to characterize the precise influence of a given test compound on the electrophysiological profile. In summary, we present a highly predictive approach for functional testing of novel GABA$_A$R related compounds for more specific and improved therapies of epilepsy, anxiety or PTSD in the future.

Material and Methods

Primary neuronal embryonic co-cultures

Primary neuronal cells from frontal cortex tissues of embryonic day 16 NMRI mice were cultured on MEA neurochips. After mechanical and enzymatic (accutase) dissociation, cells were grown in DMEM/10% horse serum without antibiotics. The networks were incubated on PDL and laminin coated MEA's (CNNS, Denton, Texas) at 37°C, with constant pH at 7.4 in a 10% CO_2 atmosphere until ready for use (generally after 4 weeks *in vitro*). Electrophysiological activity can be monitored starting from a few days *in vitro* (DIV) on for up to several months. Neurons encode information by firing action potential spikes and bursts that can be easily extracted with MEA recordings. Neuronal electrophysiology responds to transmitters, their blockers, agonists and many other pharmaceuticals in a histiotypic manner similar to the *in vivo* situation.



Left: recording set up with heater and CO_2 supply. Right: Electrical activity patterns of the simultaneously recorded spikes (action potentials) of a multitude of neurons of one cortical network.

Data Analysis

Spike train data were recorded with a microelectrode array neurochip (MEA) setup (Gramowski et al. 2004) consisting of MEA neurochips (CNNS, Denton, Texas, USA), MEA workstations (Plexon Inc., Dallas, TX, USA), and analysis software (Plexon, Inc., and NeuroProof GmbH, Rostock, Germany). Experiments were carried out with increasing concentrations, covering the full dynamic range of action. A stable activity phase of at least 30 minutes was analysed for every application phase. Chemically stimulated networks require a specific data analysis approach due to their spontaneous and complex network behaviour. To characterize the complex influence of a substance on the various network properties we calculated for each stable activity phase of a substance application 200 spike train parameters and normalized those by the native activity. The computed spike train features characterize effects on the general activity, burst structure, regularity of oscillation and synchronicity. These data records were computed for the each substance, creating a "finger print" of ist effects. All parameter values are normalized relative to the native level as an internal reference of the activity baseline.

Pattern Recognition and Classification

To clarify the mode of action of a substance on the activity of frontal cortex networks, we further processed these experiments using methods of pattern recognition. Using a feature selection algorithm, we selected the 40 most descriptive parameters of all 200 spike train parameters based on the reference substances. Feature selection is a widely accepted method in bioinformatics, and a wide range of different approaches have been proposed. We calculated the feature ranking using a MDL (minimal description length) modified algorithm that compares total correct predictions. An artificial neuronal network (multi layer feed forward network and back propagation algorithm without hidden units) was trained with the data sets of all reference substances. The respective data records of each substance was subsequently classified against the reference database.

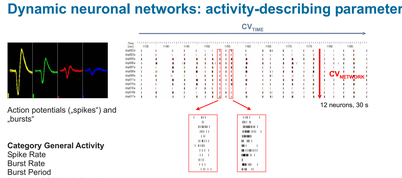
RNAi, Western Blotting and Microscopy

For **RNAi**, two lentiviruses were used containing different siRNA clones for GABA$_A$R 1 and a GFP cDNA. After transduction the cells were incubated for two weeks with two medium changes/week.

For **immunoblotting**, unboiled cell lysates from treated and untreated cultures were run on NuPage gradient SDS-gels (Invitrogen) and transferred on PVDF-membranes (iBlot, Invitrogen). Antibodies used: GABA$_A$R 1 (1:750, Synaptic Systems), SNAP25 (1:8000, Synaptic Systems). AP-conjugated anti-rabbit secondary antibodies (Invitrogen) were incubated with AP-substrate (Immobilon, Millipore) and chemiluminescence was recorded with a BioRad detection unit and quantified using Imagelab (Biorad).

Immunostaining of lentivirus-infected, fixed cells was performed with anti-GABA$_A$R 1 (1:750, rabbit & Synaptic Systems), labeled with Alexa GAR 594 (1:1000). Nuclei were stained with Hoechst (1 µg/ml). GFP+/GABA$_A$R+ cells were counted manually using a Nicon Eclipse E800 (63x objective). For quantification of receptor punctae, images were acquired using a Leica SP2 AOBS confocal laser scanning microscope. Maximum projections were binarized using ImageJ and particles counted automatically after pixel erosion and dilatation.

Dynamic neuronal networks: activity-describing parameters



Action potentials („spikes") and „bursts"

Selection of 31 (out of 200) parameters in 6 global categories:

General activity	Synchronization	Oscillation
Spike rate	Spike rate CVnet	Spike rate CVtime
Burst rate	Burst rate CVnet	Burst rate CVtime
Burst period	Burst period CVnet	Burst period CVtime
Interburst interval	Interburst interval CVnet	
Burst surprise	Burst surprise CVnet	
% of spikes in burst	% of spikes in burst	
Number of bursting units		
Burst structure	**Network variation of bursts**	**Temporal variability of bursts**
Burst duration	Burst duration CVnet	Burst duration CVtime
Number of spikes in burst	Number of spikes in burst CVnet	Number of spikes in burst CVtime
Interspike interval	Interspike interval CVnet	Interspike interval CVtime
Mean Frequency in burst	Mean Frequency in burst CVnet	Mean Frequency in burst CVtime
Peak frequency in burst	Peak frequency in burst CVnet	Peak frequency in burst CVtime

Category General Activity
Spike Rate
Burst Rate
Burst Period
Percent of Spikes in Burst

Category Burst Structure
Burst Duration
Number of Spikes in Burst
Burst Frequency

Category Synchronization
Variation within the network (CVnet) as an indicator for the strength of the synchronization

low variation => more uniform => stronger synchronization

Category Oscillation
Variation over time (CVtime) as an indicator for the strength of the oscillation

low variation => more regular => stronger oscillation