TECHNISCHE UNIVERSITÄT MÜNCHEN
Klinik für Neurologie

# The NMDA-Receptor on Fast Spiking Parvalbumin-expressing Interneurons

## Investigations on the Role of Disinhibition and its Effects on Gamma Oscillations, Cognitive Functions and Symptoms of Schizophrenia in a Mouse Model

Charlotta Rühlmann

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Vorsitzender: Univ-Prof. Dr. E. J. Rummeny

Prüfer der Dissertation:
1. Univ-Prof. Dr. B. Hemmer
2. apl. Prof. Dr. A. Berthele

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## List of abbreviations

aCSF: artificial Cerebrospinal Fluid

AMPA: 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic Acid

AAV: Adeno-Associated Virus

bp: Base Pairs

$Ca^{2+}$: Calcium

dB: Decibel

ChR2: Channelrhodopsin2

CSF: Cerebrospinal Fluid

DIO: Doublefloxed Inverse Orf

DISC1: Disrupted In Schizophrenia1

DSM-IV: Diagnostic And Statistic Manual Of Mental Disorders

EEG: Electroencephalography/-gram

ErbB4: v-Erb-a Erythroblastic Leukemia Viral Oncogene Homolog 4

EPS: Extrapyramidal System

EPSC: Excitatory Postsynaptic Current

EYFP: enhanced yellow fluorescent protein

fMRI: Functional Magnetic Resonance Imaging

FS: Fast Spiking

GABA: γ-Aminobutyric Acid

h: Hour (unit)

Hz: Hertz (unit)

ICD-10: International Classification Of Diseases

LFP: Local Field Potential

MAM: Methylazoxymethanol Acetate

MEG: magnetic encephalogram

MK-801: Dizocilpine

n: Number

NMDA-R: N-methyl-D-aspartate Receptor

PBS: Phosphate Buffered Saline

PCP: Phencyclidine

PPI: Prepulse Inhibition

PV: Parvalbumin

PSD: Postsynaptic Density

RNA: Ribonucleic Acid

mRNA: Messenger RNA

RS: Regular Spiking

SEM: Standard Error Of The Mean

SN: Substantia Nigra

VTA: Ventral Tegmental Area

WHO: World Health Organization

# 1. Introduction

We generated a mouse model to investigate

1. the glutamate hypothesis of schizophrenia and
2. the fast-spiking gamma hypothesis

and how they might be connected. In this first part, background information about the NMDA receptor, the neurobiological and psychiatric disease schizophrenia and its possible etiology, about brain waves in general, but especially gamma oscillations, and finally about cortical interneurons will be provided, reflecting current scientific knowledge and opinions.

## 1.1 The NMDA Receptor

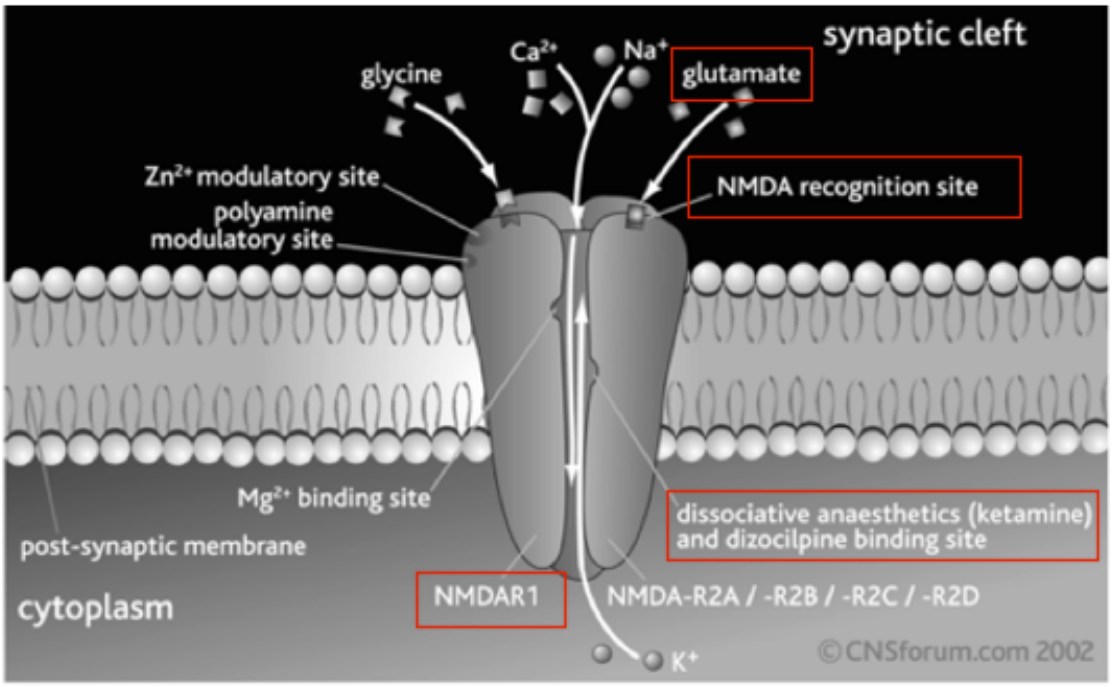
The NMDA receptor is an ionotropic glutamate receptor that can be found throughout the central nervous system, with splicing variants distinct to specific brain regions. Other receptors for glutamate are the three subgroups of metabotropic receptors and the AMPA/kainate receptors, which are also ionotropic. Glutamate is the most prominent excitatory neurotransmitter in the brain and is released into chemical synapses from vesicles stored in the presynaptic cell. It also serves as a precursor for the glutamate-decarboxylase(GAD)-catalysed synthesis of GABA in inhibitory neurons. The NMDA receptor consists of two NR1 and two NR2 subunits forming a heterotetramer. Another subunit, called NR3, seems to have an inhibitory effect on receptor activity (Cavara, Orth et al. 2010). The NR1 subunit contains a glycine-binding site while the NR2 subunit binds glutamate. Additionally to its ligand-controlled characteristics, the NMDA-R has a voltage-dependant $Mg^{2+}$-block. Only when both its ligands glutamate and glycine bind to the receptor and at the same time depolarization removes the $Mg^{2+}$-block, the ion channel opens, so that a $Ca^{2+}$-flux into the cell can take place and lead to further intracellular action. The pore is also permeable for other cationic molecules,

Sodium and Potassium. The NMDA-R plays an important role in synaptic plasticity, memory and learning, showing complex interaction with the AMPA receptor (Li and Tsien 2009).

### 1.1.1 NMDA-R Antagonists

There are specific binding sites for NMDA-R-antagonists, which are of particular interest in this context. Ketamine, MK-801 and PCP are some of the well-studied antagonists – they bind non-competitively within the ion channel and result in closing of the pore. Those NMDA-R-antagonists are used as anesthetic drugs in veterinary medicine, but also as recreational (illicit) drugs for their dissociative effect. Studies have shown that low doses of the mentioned substances induce hyperactivity of cortical pyramidal neurons (Jackson, Homayoun et al. 2004) as well as net cortical excitation as reflected in fMRI changes (Breier, Malhotra et al. 1997). This implies that general activation of NMDA-Rs has an inhibitory influence on pyramidal cells, and that blocking the receptor with antagonists would cause a state of disinhibition. Furthermore, these antagonists can induce symptoms of schizophrenia and worsen the clinical symptoms of schizophrenic patients (Rujescu, Bender et al. 2006), (Lindsley, Shipe et al. 2006), leading to the "glutamate hypothesis" of schizophrenia.

**Fig. 1.1** Scheme of the NMDA receptor in the cell membrane of a postsynaptic cell (NR3 subunit not shown)




*(Modified from CNSforum.com)*

## 1.2. Symptoms and Etiology of Schizophrenia

Since no molecular abnormality has been identified that could define schizophrenia, the diagnosis is based on the presentation of a very distinct pattern of symptoms. Commonly, those symptoms are divided into three groups: The positive and negative symptoms as well as cognitive impairments. Positive symptoms include hallucinations, delusions, and bizarre thoughts, while negative symptoms can present as flat affect, social withdrawal and apathy. Cognitive impairments have long been thought to result from positive symptoms, but they should be considered an individual group of symptoms. Disorganized thinking and speech are the strongest part of the cognitive impairments, accompanied by deficits in working memory and problems in executive functions. Negative symptoms resemble aspects of depression. It is estimated that 1% of the world population suffers from schizophrenia, which can lead to disastrous situations in countries without the means to support patients and their caretakers. Lifetime occurrence of substance abuse in

schizophrenic patients is almost 50%, the incidence of comorbidities is high and social problems like homelessness and poverty are common. In the United States, DSM-IV criteria are used for diagnostic means, while in European countries WHO's ICD-10 gives the basis for diagnosis and classification in subforms of the disease. Both systems require characteristic symptoms of all three groups persisting for more than six months and social or occupational dysfunction of the suffering person for the diagnosis of schizophrenia. Subforms are the paranoid type, the disorganized or hebephrenic type, and the catatonic form, where patients characteristically exhibit catatonic stupor and wave flexibility. A mild form is called schizophrenia simplex, while post-schizophrenic depression and residual schizophrenia describe lasting partial symptoms after decay of other symptoms.

In search for the etiology of schizophrenia there is currently a tremendous work being done in the field of genetics, aiming to identify risk genes for the disease. Some of these genes of interest have been exposed in studies of affected families, others were found through genetic analyses of tissue from schizophrenic patients and their relatives from various countries and all continents. DISC1 is short for Disrupted In Schizophrenia; mutations in this region (a balanced translocation leading to disruption of a gene at 1q42) were first discovered in a Scottish family with an extremely high incidence of psychiatric diseases like schizophrenia, bipolar disorder and depression, which is remarkable since those diseases have overlapping symptoms, although each has its own diagnostic description (Millar, Christie et al. 2001). Latest research shows evidence that DISC1 controls wnt/GSK3-ß signaling and brain structure by decreasing neural progenitor proliferation (Singh, De Rienzo et al. 2011). Neuregulin 1 (NRG1) might be another susceptibility gene, located on chromosome 8 and encoding for a growth factor that suppresses NMDA receptor activity via interactions with the receptor tyrosine-protein kinase ErbB4 (Stefansson, Sigurdsson et al. 2002). Another candidate gene is one that got into focus of research by genome-wide linkage analysis, the gene encoding for the neurotransmitter-degrading enzyme Catechyl-O-Methyltrandferase (COMT) on chromosome 22. Most of the genes in focus are involved in

synaptogenesis or neuroplasticity via interactions with dopaminergic, glutamatergic or GABAergic signaling. DISC1 and ErbB4 pathways might be functionally convergent in regulating the postsynaptic density (PSD) of excitatory synapses, DISC1 interfering with the wnt-pathway and GSK3β signaling, which in turn regulate gene expression and, through this, apparently brain development. Genetic studies have also found a connection between PDE4A and PDE4B and schizophrenia, both proteins that hydrolyze cAMP and have been shown to be involved in learning and memory (Pickard, Thomson et al. 2007). Another recent approach found different immune-related susceptibility genes for schizophrenia (Stefansson, Ophoff et al. 2009). In search for the cause, there is thus evidence for different mutations in several genes, each of which seem to be causing disruptions in one or more neurotransmitter systems and which affect neurodevelopment and synaptic plasticity. A closer look reveals that these genes seem to affect mental health, but also mood and memory function. Schizophrenia has numerous variants and some symptoms resemble other psychiatric diseases very much.

A century ago, Wernicke contemplated that schizophrenia might be caused by disruption of association fibers, connecting different areas of one hemisphere. More recently, the "disconnection hypothesis" described the idea that schizophrenia could be characterized or even caused by disrupted signaling pathways between brain regions or cellular circuits (Stephan, Baldeweg et al. 2006). This functional disconnection could be caused by structural changes in axonal projections or by disturbed neurotransmitter signaling, which each can have a multitude of origins. Furthermore, it has been hypothesized that viral infections during pregnancy and also during childhood, maybe through subtle developmental impairments, could be risk factors for the developing child to suffer from schizophrenia in adulthood. Environmental influences during early childhood seem to play a role and the use of drugs that interfere with the endogenous reward system, especially cannabis, can be a trigger for the disease (Arendt, Rosenberg et al. 2005) .

One gets the impression that risk genes account for the first level in the fate of schizophrenic patients – whether a person who carries these mutations becomes schizophrenic or not could then depend on their environment and their lifestyle, mediated through an (im-) balance of neurotransmitters and cortical circuit functioning. The molecular etiology remains unclear and most of the findings give rise to new questions about the understanding and definition of the disease. The relatively new field of epigenetics could become of enormous importance in understanding how lifestyle, environment and a person's biographical experience all contribute to changing the neurobiological architecture and the manifestation of a disease.

### 1.2.1 Pharmacological Treatment of Schizophrenia and the "Dopamine Hypothesis"

Schizophrenia has over decades been treated with D2-receptor antagonists, known as typical antipsychotics or classical neuroleptics. High-potency example of this class is Haloperidol, which replaced Chlorpromazin, the very first antipsychotic drug. By interference with the dopamine system in different subcortical regions, Haloperidol causes strong extrapyramidal side effects, also called Pseudo-Parkinson or Parkinsonoid. Atypical antipsychotics like Clozapine, Olanzepine and Risperidone have fewer side effects on the EPS and some of them are not only approved for treatment of schizophrenia, but for indications as bipolar maintenance, acute mania, and bipolar depression – they also relieve negative symptoms of schizophrenia, unlike the earlier drugs. Like Haloperidol, atypical antipsychotics also influence the dopamine system, but the single substances vary in their affinity of dopamine receptor types. The "dopamine hypothesis" for schizophrenia evolved from observations in clinical and experimental settings which showed that blockage of D2 dopamine receptors can ameliorate the positive symptoms while amphetamines, through an increased release of dopamine in limbic areas such as nucleus accumbens and in the prefrontal cortex, lead to exacerbation of symptoms in schizophrenic patients (Sawa and Snyder 2002). Regarding the role of dopamine in the

reward system and the high incidence of schizophrenia in drug-abusers, there seems to be evidence for an imbalance of dopamine in the schizophrenic brain. And yet, Haloperidol is not completely selective to the D2 dopamine receptor, but it also influences NMDA receptor expression (Gardoni, Frasca et al. 2008). Also, dopaminergic neurons (for example in the substantia nigra (SN) and in the ventral tegmental area (VTA)) receive glutamatergic inputs. Which leads to a connection between another neurotransmitter system and back to the "glutamate hypothesis", mentioned before (see 1.1.1). The two systems can hardly be separated and investigated on their own – in vivo, there are downstream effects in the other system, respectively, some of which are not fully understood. Maybe the terms "dopamine hypothesis" and "glutamate hypothesis" are too simple to describe the consequences of any impairment in either of these systems. Dopamine receptor antagonists like Haloperidol or Chlorpromazin show their clinical effect after a period of days or even weeks, even though the molecular effects take place within few hours. Neurochemical, structural and epigenetic mechanisms could occur in between and lead to changes in electrophysiological properties and synaptic signaling.

## 1.3 Gamma Oscillations and Schizophrenia

Another clinical finding in schizophrenic patients is an unusual pattern of gamma oscillations in EEG recordings (Spencer, Nestor et al. 2003). Gamma oscillations are fast waves of coordinated neuronal activity around a frequency of 40 Hz that can be evoked by mental tasks and seem to be critical for the integration of stimuli into conscious perception. Gamma oscillations were first detected in the olfactory bulb of hedgehogs, following olfactory stimulation. Cortical gamma oscillations are thought to represent vigilance, attention and alertness (Fries, Reynolds et al. 2001). Apparently, schizophrenic patients have higher general baseline amplitudes of gamma oscillations, but they fail to show an appropriate increase in this range of oscillations when challenged with a task that reliably elevates the gamma amplitude in healthy control subjects (Kissler, Muller et al. 2000). Brain waves in general have been examined for decades and from different perspectives. One basic requirement for

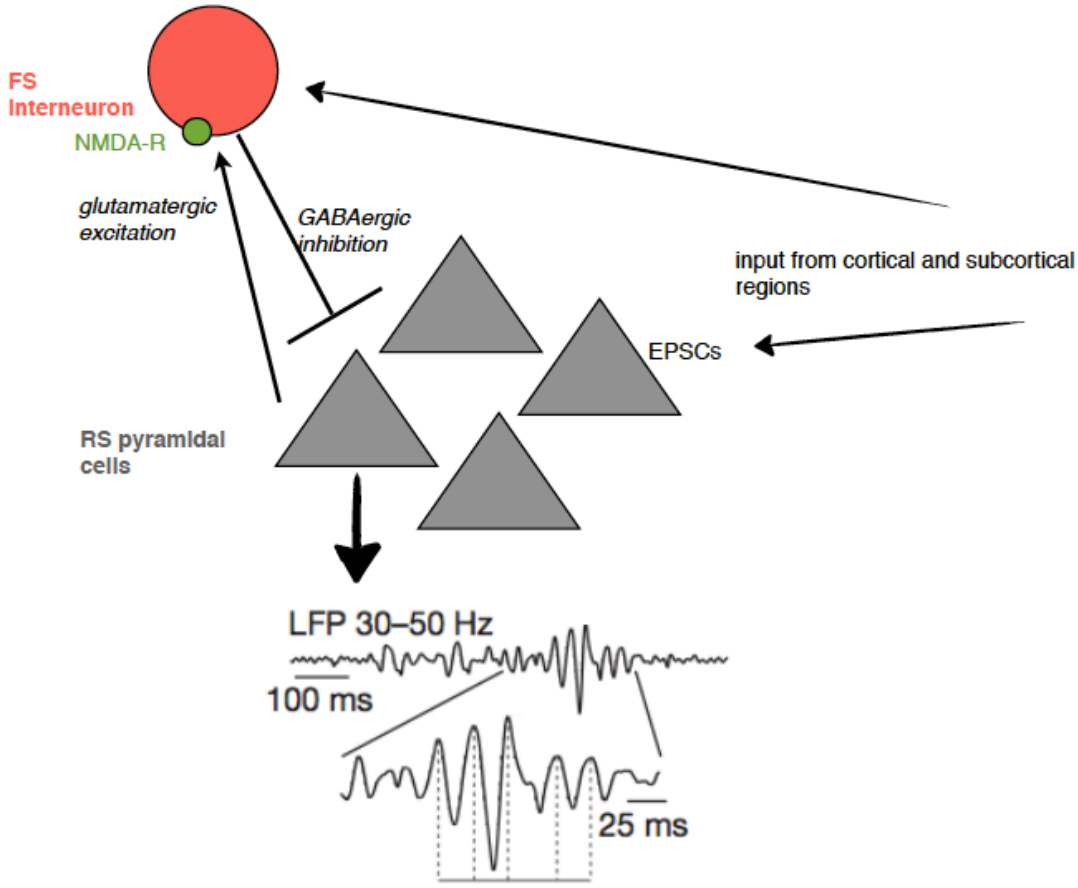
populations of neurons to generate oscillations, which may be involved in cognitive functions and communication between cortical structures as well as coordination of population activity, is synchrony. Correlated neuronal activity is one form of synchrony, occurring without or within oscillations. Having started with gamma oscillations, there are, at the other end of the range of frequencies, very slow waves in a frenquency of <1 Hz, also called up- and downstates. These reflect population changes in membrane potential, which, similar to the gamma cycle, determine whether stimuli will be transmitted or not. It is postulated that neurons can only fire when in an upstate, a depolarized state of ~ -65 mV. Upstates, propagating as waves over large areas, have been shown to originate in specific cortical layers (Stroh, Rühlmann et al., manuscript to be published in Neuron®), especially layer V. Between the very fast and the very slow patterns of brain activity lie the frequencies that reflect specific phases of alertness, sleepiness and sleep itself. Electrical neuronal activity critically depends on synaptic excitation and inhibition, bringing neurotransmitters and their receptors into the play of organized brain activity and cognitive function.

### 1.3.1 The Gamma Cycle

The "gamma cycle", first described by W. Singer (Fries, Nikolic et al. 2007), is a model for how networks of excitatory and inhibitory neurons produce synchrony and precision in spike activity, which is necessary for example in memory processes and sensory-motor-coordination. These neuronal networks generate synchronized oscillations in the gamma band via synaptic inhibition and supposedly via gap-junctional coupling between interneurons and pyramidal cells, but more importantly between clusters of interneurons that are electrically coupled (Hormuzdi, Pais et al. 2001). Whether or how an excitatory postsynaptic potential (EPSC) affects an excitatory neuron in this network seems to be determined by the phase of the gamma cycle in which it arrives at the target cell, meaning the strength of inhibition from the interneurons. The interneurons, in turn, are activated by glutamatergic signaling from those pyramidal cells - and the idea of a circle describes that

process very well. Oscillatory activity of pyramidal neurons is thus tuned by a cycle of inhibition from interneurons which they activate themselves, and it leads to discrimination of both strength of input and the timing of it, happening for example with thalamic input following sensory stimulation. Another picture would be a group of interneurons and several groups of excitatory neurons in different brain regions, all projecting to a "target" group of pyramidal cells. The interneurons fire in a rhythmic and cyclic manner, providing stronger and less strong inhibition. This inhibition determines which input will be transmitted, but also how fast this will happen. This hypothesis of coding strategy supports a fast and flexible way of processing input. Still, it is only one possible explanation of network properties and covers some, but not all aspects of rhythmic synchronization.

Figure 1.2 **Simplified illustration of the gamma cycle, showing only somata of pyramidal neurons and interneurons**




*Inhibition from fast spiking interneurons follows a wave-shaped pattern and determines the further networt activity following sensory or other input.*

Equivalent to the gamma cycle in cortical areas, activity of hippocampal "place cells" (neurons in actively exploring rodents, which respond to a specific region in the animal's environment) seems to depend on a theta cycle. The resulting frequency of 4 – 8 Hz is called "theta-phase procession" and is resulting from inhibition provided by interneurons.

## 1.4 Interneurons

Consistent with findings of other groups, we found proof that fast-spiking (Stefansson, Sigurdsson et al.) interneurons play an essential role in the modulation of gamma oscillations in the rodent brain. Basic evidence for the relationship between interneurons and pyramidal cells was provided by Buszaki and colleagues, who used hippocampal slices to demonstrate that pyramidal cell firing preceded firing from interneurons by milliseconds (Csicsvari, Jamieson et al. 2003). In a previous project, we used a PV-Cre knock-in mouse line to test the fast spiking gamma hypothesis (Cardin, Carlen et al. 2009). Using an adeno-associated virus (AAV) to induce a Cre-dependant and therefore cell-type-specific expression of Channel-Rhodopsin (ChR2), an ion channel that can be activated by light, we performed local field potential (LFP) recordings, simultaneously with laser-activation of the virally transduced cells. LFP recordings measure electrophysiological signals resulting from the sum of all dendritic activity within a volume of tissue. In that aspect, they can be compared to EEG recordings, since they do not measure activity of single cells, but they intracranially monitor the overall electrical activity (currents) of multiple neurons, both subthreshold and spiking activity, in a spatially more confined manner than EEG. Those recordings revealed that "...light-driven activation of fast-spiking interneurons at varied frequencies (8-200 Hz) selectively amplifies gamma oscillations. In contrast, pyramidal neuron activation amplifies only lower frequency oscillations."

Interneurons are mostly multipolar neurons that connect afferent and efferent

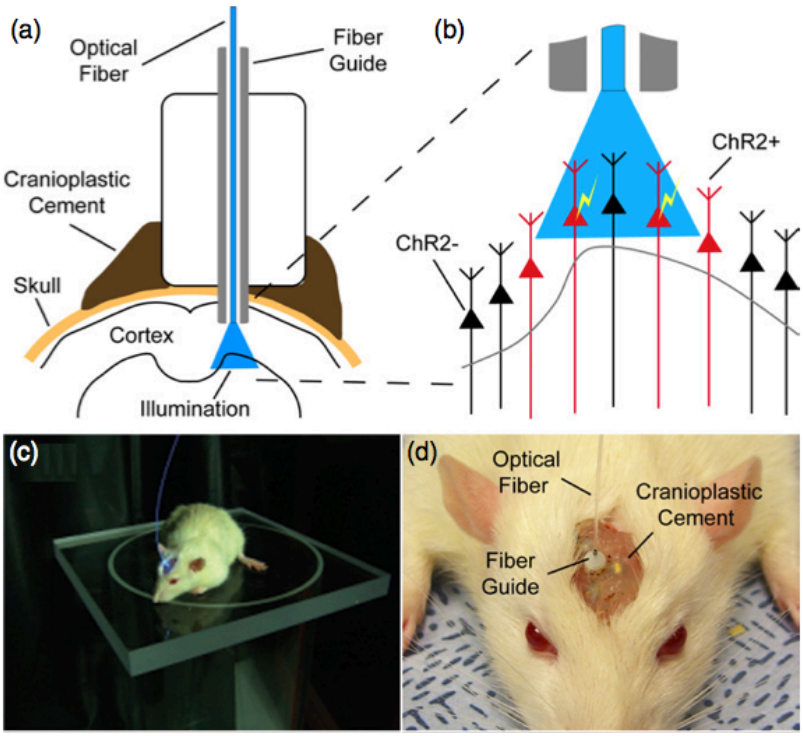
neurons in brain circuits. They make up 10-20% of the neurons in neocortex and they are typically GABAergic and therefore inhibitory. Generally, there is a delicate balance between excitation and inhibition in the brain, which needs to be permanently controlled and maintained in order to enable fast signaling but prevent overexcitation, which could lead to neurotoxicity or uncontrolled firing and seizures. Some of the GABAergic interneurons in the cortex, predominantly basket- and chandelier-cells, express the Calcium-binding protein Parvalbumin (PV). Other markers for classes of interneurons, which vary in morphology and physiology, are calretinin and calbindin, and the neuropeptides Somatostatin (SST) and Neuropeptide Y (NPY). Basket cells form synapses close to the soma of their target cells, while SST-expressing cells have their synapses at distal dendrites of their receiving cells. This gives rise to the idea that interneurons can either control the "output" from or the "input" to pyramidal neurons. PV-interneurons, the largest population of cortical interneurons, exhibit a fast-spiking firing pattern and have glutamate receptors. Especially in layers II/III and V of somatosensory and frontal cortex, these cells show high density and presumably unspecific connectivity to local pyramidal neurons, providing a "blanket of inhibition" as a "canonical feature in the design of neocortical microcircuits" (Packer and Yuste 2011). Post-mortem studies have shown decreased PV-expression in the prefrontal cortex (PFC) in brain tissue of schizophrenic patients (resulting from a reduction of measurable PV-mRNA per cell, not a decrease in the number of PV-positive cells) (Benneyworth, Roseman et al. 2011), (Torrey, Barci et al. 2005).

## 1.5 ChannelRhodopsin (ChR2)

Optogenetics are a powerful tool for activation of a specific population of neurons in anesthetized or awake animals. For this approach, viral injections into specific areas of transgenic (Cre-expressing) animals lead to expression of ChR2 and a fluorophore, in our case mCherry, which makes them a target for photostimulation and also allows post-mortem analysis of stimulated populations. ChR2 is a rhodopsin and ion channel with a microbial-type all-trans retinal chromophore, which originally stems from the green algae

Chlamydomonas reinhardtii. It reacts upon absorption of a photon (in experimental settings coming from a laser with specific wavelength) with opening of the channel, which is selective for mono- and divalent cations and thus, when activated, leads to depolarization of the membrane potential and reliable induction of a photocurrent that can be measured in patch-clamp experiments (Nagel, Szellas et al. 2003). 10 days post-injection, virus expression is strong enough to carry out experiments. A laser beam with the suiting wavelength for induction of the conformational change is then guided through a fiber to the surface above the desired brain area (Zhang, Wang et al. 2006), (Nagel, Szellas et al. 2003). Optogenetics are hence a well-suited tool to examine the net effect of specific activation of just one class or population of neurons. It is also possible to "rescue" activity of otherwise dysfunctional neurons.

**Fig 1.3** Optogenetic activation of virally transduced neurons




*(Adapted from (Aravanis, Wang et al. 2007), Fig. 1 "Overview of the optical neural interface")*

**a** *Optical fiber inserted into fiber guide and blue light transmitted to the cortex.*
**b** *Close-up schematic of the stimulated region (here, pyramidal cells are targeted).*
**c** *Blue light is transmitted to target neurons via the optical fiber in a rat.*
**d** *Close-up view of the fiber guide attached with cranioplastic cement.*

From observations in clinical and laboratory studies, the hypothesis arises that some distinct symptoms of schizophrenia might be caused by an imbalance of neuronal activity. This could be caused by disrupted neurotransmitter-mediated circuit functioning, or in other words: by a pathological state of disinhibition between inhibitory interneurons and excitatory neurons.

An interneuron releases the neurotransmitter GABA and thereby inhibits excitatory, glutamatergic cells. If, however, the interneuron has a dysfunctional NMDA-receptor that makes it impossible for the cell to respond with activity,

meaning GABA-release, to ligand binding, the efferent (and at the same time afferent, see fig. 1.1) neuron will not be inhibited. The fast-spiking gamma hypothesis is based on the thought that fast-spiking interneurons create a narrow window for effective excitation - and the production of gamma oscillations - by synchronous inhibition of excitatory cells (Cardin, Carlen et al. 2009). This principle seems to be an important system of network coordination in the healthy brain. We investigated the model of disinhibition caused by NMDA-R-dysfunction on PV-expressing interneurons by testing transgenic mice in electrophysiological and behavioral paradigms with and without pharmacological treatment targeting the NMDA receptor.

# 2. Materials and Methods

## 2.1 Genetic Ablation of NMDA-R Activity in PV-Interneurons

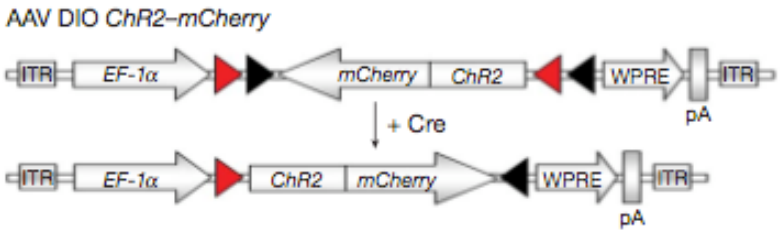
Using Cre-loxP technique, we created a conditional knockout of the NR1 subunit of the NMDA receptor, specific for PV-expressing interneurons. Cre-recombinase is driven by a promotor, in this case PV, which makes it tissue- and cell-type-specific and determines when the knockout will be effective (only after the gene is expressed). For this mouse line, a crossing between a PV-Cre mouse and one that carries two floxed alleles of the gene of interest is necessary. Floxed means that the gene that is supposed to be knocked out is in both directions flanked by the same palindromic sequence, 13 bp long, which will be recognized by the recombinase and cut out in the Cre-expressing cells. This results in a postnatal cell-type specific knockout of the NR1-subunit of the NMDA receptor. The offspring will then have two genotypes: PV-Cre+, NR1 f/f or PV-Cre-, NR1 f/f. The mice that did not express Cre and thus had normal NMDA receptors were our control group for behavior experiments. Cre-positive mice without the floxed NR1 gene were the control animals in electrophysiological experiments involving optogenetic activation.

## 2.2. Virus Injections

An adeno-associated virus (AAV) was generated to transduce Cre-expressing PV interneurons. This virus contains a double-floxed inverted reading frame and enables us to achieve Cre-dependant expression of Channel-Rhodopsin (ChR2) and mCherry (a red fluorescent molecule) only in PV-interneurons. ChR2 fused to the fluorescent protein mCherry was cloned in antisense direction into pAAV-MCS (Stratagene) to create AAV DIO ChR2-mCherry (see Fig. 2.1). The virus was injected into the desired brain area in a stereotactic surgery after the mouse was anaesthetized with an intraperitoneal injection of a mixture of ketamine (1.1 mg/kg) and xylazine (0.16 mg/kg). A small

craniotomy was then made 1.5 mm posterior to the bregma and 3.0 mm lateral to the midline above barrel (somatosensory) cortex. The virus was then delivered through a small durotomy by a glass micropipette attached to a Quintessential Stereotaxic Injector (Stoelting). The glass micropipette was lowered to 0.4 mm below the cortical surface. A bolus of 0.5 μl of virus (AAV DIO ChR2-mCherry; 2 x $10^{12}$ viral molecules per ml) was injected into barrel cortex at 0.1 μl/min. The pipette was then retracted to a depth of 250 μm below the surface and an additional 0.5 μl virus was injected at the same rate. The pipette was held in place for 5 min after the injection before being retracted from the brain. The scalp incision was sutured, and post-injection analgesics were given to aid recovery (0.1 mg/kg Buprenorphine).

**Fig. 2.1** AAV DIO ChR22mCherry

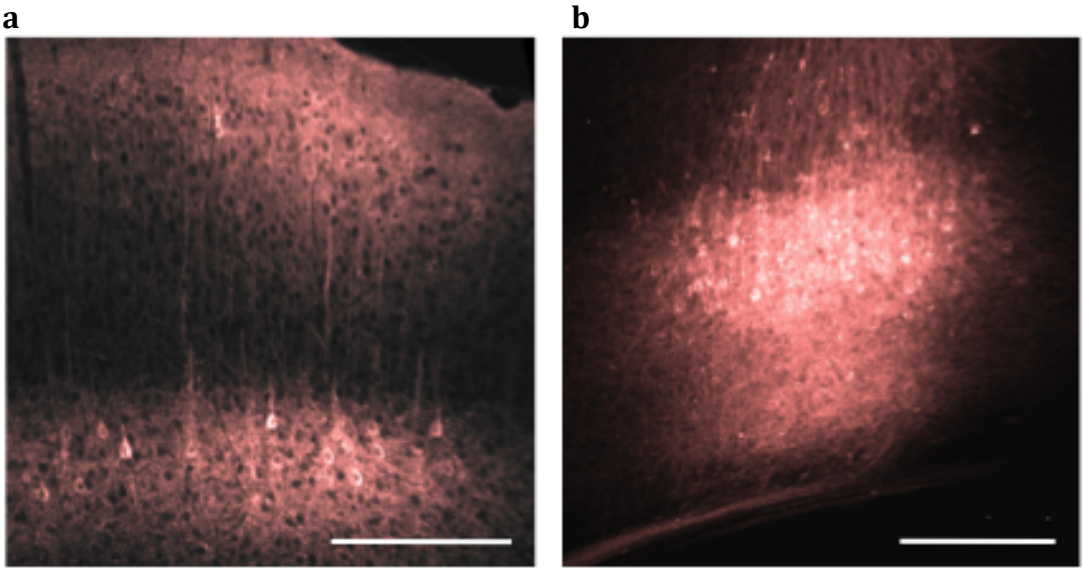



*(Adapted from Cardin, Carlén et al. 2009)*

*Cre-dependent expression of ChR2 enabled cell-type-specific targeting of light-activated channels. In the presence of Cre, ChR2-mCherry is inverted into the sense direction and expressed from the EF-1a (EEF1A1) promoter. (ITR = inverted terminal repeat; pA = poly(A); WPRE = woodchuck hepatatis B virus post-transcriptional element).*

One to three weeks after the viral injections, electrophysiological recordings were performed (maximal recombination can be observed one week to ten days post-injection and the expression of the virally delivered genes stays constant for a time period of at least seven months, see Fig. 2.2).

**Fig. 2.2** ChR2 expression in virally transduced cortical pyramidal neurons in V1




*Expression of ChR2-mCherry in virally transduced pyramidal neurons in layer V of the visual cortex of mice (V1). Unpublished data from C.R. obtained in A. Konnerth's laboratory.*

**a.** *20 x magnification (plus zoom) confocal image of virus expression in a sagittal slice 10 d post-injection. Scale bar 100 μm.*
**b**. *20 x magnification confocal image (stack) of virus expression in a coronal slice 7 months post-injection. Scale bar 100 μm.*

## 2.3 Electrophysiology

For the non-awake experiments, mice were anesthetized with isoflurane and held in place with a head post cemented to the skull. All incisions were infiltrated with lidocaine. A small craniotomy was made over barrel cortex approximately 200 μm anterior to the virus injection site. Extracellular single-unit and LFP recordings were made with tetrodes or stereotrodes. Intracellular recordings were conducted by whole-cell in vivo recording in current clamp mode. Stimulus control and data acquisition was performed using software custom-written in LabView (National Instruments) and Matlab (The Mathworks).

### 2.3.1 Light Stimulation

Light stimulation was generated by a blue laser of 473 nm wavelength (Shanghai Dream Lasers), which was controlled by a Grass stimulator (Grass Technologies) or computer. Light pulses were given via a 200-μm diameter, unjacketed optical fiber (Ocean Optics) positioned at the cortical surface 75-200 μm away from the recording electrodes.

### 2.3.2 Data Analysis

Unit and LFP analysis used software custom-written in Igor Pro (Wavemetrics). For each stimulation frequency, we measured the relative power in an 8-Hz band centered on that frequency. For each recording site, we measured power from 5 to 10 LFP traces under each condition. Example power spectra are averages of the power spectra from 5-10 traces of unfiltered LFPs from individual experiments. Relative power was calculated by measuring the ratio of power within the band of interest to total power in the power spectrum of the unfiltered LFP. We also measured the power ratio: $P_{light}/P_{baseline}$, where $P_{light}$ is the relative power in a frequency band in the presence of light stimulation and $P_{baseline}$ is the power in that band in the absence of light stimulation. Statistical significance was assessed with the Mann–Whitney test (between-group comparisons) and the Wilcoxon signed-rank test (within-group comparisons).

### 2.3.3 Awake Electrophysiology

A total of 16 animals were used for awake electrophysiology (8 for each genotype). Teflon-coated tungsten electrodes were implanted under ketamine/xylazine anesthesia. Two electrodes were placed bilaterally in barrel cortices, 1.5 mm posterior to bregma and 3.5 mm from the midline. In each hemisphere, a signal electrode was implanted 0.5 mm below the cortical surface and a reference electrode was implanted 1.75 mm below the cortical surface. A stainless steel screw on the right posterior parietal cortex served as ground. Animals were allowed to recover for 4-5 days before recording.

Recording sessions took place in an empty box (L30 cm x W19 cm x H13 cm), to which animals had not been previously exposed. After a 5-10 minute habituation period, 20 minutes of baseline data were recorded for each animal. After the baseline period, animals were injected intraperitoneally with 0.5 mg/kg of the NMDA-R antagonist MK-801, and another 40 minutes of data were recorded. Statistical significance was assessed with the Wilcoxon rank-sum test (between-group comparisons) and the Wilcoxon signed-rank test (within-group comparisons). Gamma events were found by filtering data between 30 and 50 Hz (126th order FIR filter) and taking the Hilbert transform to compute the "envelope". A 1 second moving average window was applied to the envelope, and a "gamma epoch" was said to occur when the envelope remained above the mean for a predetermined amount of time. The data from two animals (one of each genotype) were contaminated with 60-Hz noise, presumably due to improper grounding. These animals were excluded from the analysis. "Relative power" indicates spectral power as a proportion of overall power between 1 and 100 Hz. "Normalized relative power" indicates relative power normalized to the mean power during the 10-minute pre-drug baseline interval. The published paper (Carlen, Meletis et al. 2012) contains parts of this methodological description, plus additional information on the electrophysiological experiments carried out by members of the Moore Lab.

## 2.4 Behavioral Tests

With another group of mice, we performed behavioral tests to assess the paradigms that are currently the standard measurements in animal models for psychiatric research in neuroscience. With only male control and conditional knockout mice, we performed the Open Field Test (VersaMax, AccuScan Instruments), which represents a fully automated measurement of the animal's behavior in a novel environment. We tested prepulse inhibition (PPI) and the startle response using Kinder Startle Monitor Systems, and we set up a T-Maze and performed the well-established Morris Water Maze as well as the Fear Conditioning Test. Behavioral parameters were analyzed for statistical means by t-tests comparing the two genotypes, except when otherwise noted. $P < 0.05$

was considered significant. All results are presented as mean ± standard error of the mean.

### 2.4.1 Open Field Test

The Open Field Test consists of a 42 cm x 42 cm x 30.5 cm (W x D x H) box, closed tightly and equipped with light and a fan for ventilation. 16 laser beams across the floor, spaced 1.0" apart, measure every movement of the mouse and give the researcher insight into an animal's general activity, whether it displays stereotypic movements and how much time it spends in the center of the box versus in the margins. Mice were 7 weeks old when first tested and 11-12 weeks old when tested again.

### 2.4.2 Prepulse Inhibition

Prepulse Inhibition is a method that is used in animal as well as in human studies. It is based on the principle that a stimulus (in this case a tone) produces a response (in this case a startling movement). If the stimulus is supplemented by a weaker tone shortly before the actual tone, the response to the second one should be diminished. This habituation to a stimulus is considered a very simple and unconscious form of learning and it is in experimental settings disrupted by NMDA-R antagonists (Klamer, Palsson et al. 2004). Deficits in this system have been linked to abnormalities in sensorimotor gating and are thought to reflect the inability to filter out unnecessary information. Studies have been published that find PPI deficits in schizophrenic patients; others find no difference between schizophrenic patients, their unaffected siblings and healthy control subjects (Roussos, Giakoumaki et al. 2011). Regardless of the debate around this test, it is currently used in most schizophrenia models, it is reliable since it measures truly blindly (because it is fully automatic and very precise as long as the researcher is aware of the need to calibrate the system), and it is one of the few tests that can be performed in both human studies and animal models. We used a system that tests for startle response and PPI after two days of acclimation (5

minutes each) to the instruments. There is a background tone, absolutely no sound can be heard from outside the box, and the box is dimly lit. The response to the tone is measured by a piezoelectronic sensor platform, and displayed as force in Newton (N). On the first day after acclimation, PPI testing day, each animal was exposed to 65 dB ambient noise for 5 minutes followed by the testing session. The PPI paradigm consisted of trials with presentation of a startle stimulus alone and trials where a prepulse of varying intensity preceded the startle stimulus by 100 ms. A total of six blocks were presented in a session and corresponding responses were averaged for each mouse and trial separately. The maximum response in Newton (N) within 65 ms post-stimulus was utilized as the startle amplitude. On the second day of the experiment, the startle response to different dB-levels was measured. This is of enormous importance to later on compare PPI deficits, because it assures that two groups of mice differing in their genotypes have the same response curve, and no motor or hearing impairments. Around a pseudo-randomly sorted trial of pulses, one set of four times a constant tone of 120 dB is presented in the beginning, and the same set of tones is presented before the end of the experiment. This subset of tones is used to compare different genotypes' phenotypes of habituation, because any healthy organism would significantly decrease the strength of the response from the first set to the second set of tones. Schizophrenic patients have been shown to show significantly less habituation in this simple test than healthy control subjects. Habituation (%) was then calculated using the equation (100–(mean startle block 2/mean startle block 1) x 100)).

The acoustic startle response (ASR) was calculated as the mean startle amplitude of the pulse-alone trials. The percent PPI was calculated using the following equation: [100-(mean prepulse response/mean ASR) x 100]. The acoustic startle threshold was assessed in the same apparatus by determining the startle response to increasing sound intensities (70, 75, 80, 85, 90, 95, 100, 105, 110, 115 and 120 dB), presented in six blocks in a fixed pseudo-randomized order.

### 2.4.3 Fear Conditioning

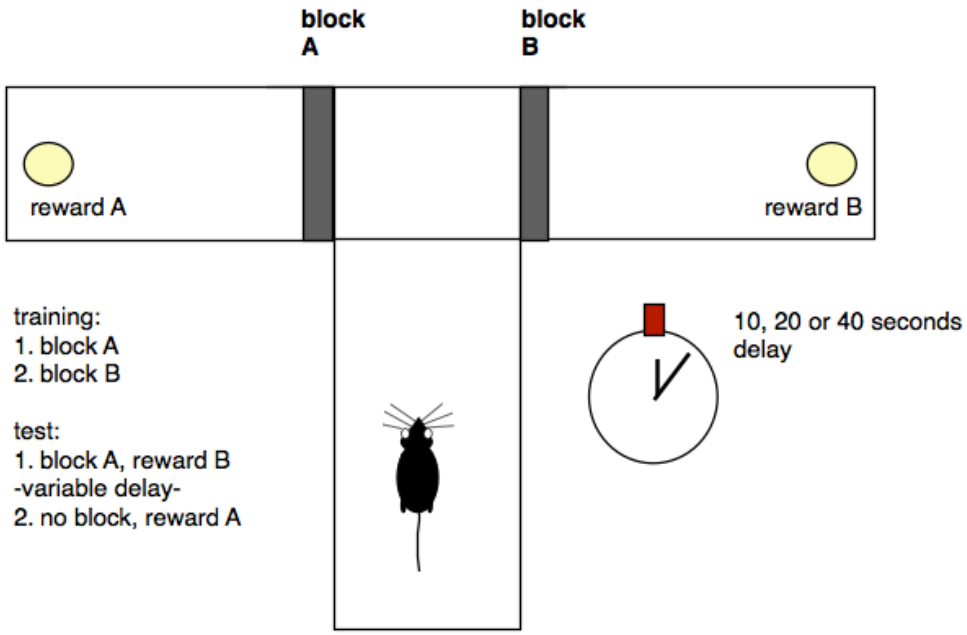
To evaluate the memory function of PV-Cre/ NR1 f/f mice, we exposed them (n = 5 for each genotype) to cued and contextual fear conditioning paradigms. Mice were put on a grid that would give them electrical shocks after they heard a tone (only in the cued version). In this setup, a neutral stimulus (the tone or the context) is the conditioned stimulus, the aversive stimulus (the shock) is the unconditioned stimulus, and the fear is the conditioned response to the pain that is caused by the shock. In the second part of the cued experiment, the sound was presented without the following shock and a researcher observed the freezing of the mice, which is thought to represent a reaction to the memory of the sound which was formerly associated to a foot shock. In the contextual version of the experiment, the mouse is tested for its memory of the environment where it first experienced the painful foot shock.

### 2.4.4 T-Maze

The T-Maze that we used is called “discrete paired-trial variable-delay T-maze task” (Aultman and Moghaddam 2001) and is used to test working memory after an initial training. For 21 days, the mice were in parallel food-restricted and habituated to the maze, thus learning that they would find reward in the end of both arms of the T-shaped maze. A loss of ~10 % of body weight is regarded necessary for the mice to be motivated and consume the reward consisting of sweetened condensed milk. Habituation was followed by a first phase of training, where first one arm of the maze is blocked, then the other one. In the next phase, a forced run is followed by a choice-run, with a short intratrial delay of <10 s. Here, the learning process starts. First, the mouse finds reward in the unblocked arm, but then, after the intratrial delay of 10 seconds, where the mouse is elevated from the maze, reward can only be found in the previously blocked arm. Once all the mice achieve 70% of right choices (meaning that they always chose the previously blocked arm) the actual experiment takes place and the percentage of correct choices is observed. We tested both groups for 3 different delays: 1 s, 20 s and 40 s, ten times per day

per mouse.

**Fig. 2.3** Principle of the discrete paired-trial variable-delay T-maze task




### 2.4.5 Morris Water Maze

To test for spatial memory abilities, we performed a Morris Water Navigation Task. In this experiment, mice have to find a hidden platform in a small pool of water, with visual cues, such as colored shapes placed around the pool in plain sight of the animal. After 8 days of training, the escape latency, which is the time needed to find the platform, was measured in a probe trial on day 9. To address reversal learning, we introduced the hidden platform in a different quadrant and continued the learning paradigm for 4 days, followed by another probe trial on the 5$^{th}$ day.

## 2.5 Immunohistochemistry

For histological evaluation, mice were transcardially perfused with 100 mM PBS followed by 4 % formaldehyde in PBS, and brains were post-fixated for 18 h at 4° C. Free-floating sections of 30 μm were cut using a vibratome (Leica VT100) and incubated with blocking solution (10% donkey serum in PBS with 0.3% Triton X100) for 1 h at room temperature (20° C) and then incubated at room temperature overnight with primary antibody diluted in blocking solution. Secondary antibodies were applied after washing with PBS and incubated for 1 h at room temperature. Sections were mounted on glass slides with Vectashield (Vector Laboratories) and coverslipped. For quantification and imaging, a Zeiss LSM510 confocal microscope was used.

Cre recombination was quantified in PV-Cre mice crossed to the R26R-EYFP Cre-reporter mouse line. Sections were stained for PV and co-expression of secondary antibody and reporter EYFP was analyzed.

## 2.6 Pharmacological Manipulation

We investigated brain rhythms and behavioral paradigms of both groups with and without pharmacological treatment. The NMDA-R antagonist MK-801 was injected intraperitoneally in a low dose of 0.3 mg/kg in behavioral experiments and 0.5 mg/kg for electrophysiological experiments. In the open field test and in LFP recordings, we started the experiment without the drug, interrupted, gave the injection, and immediately began the second part of the experiment.

## 2.7 Slice electrophysiology

PV-interneurons were indentified by the expression of the mCherry fluorescent protein.

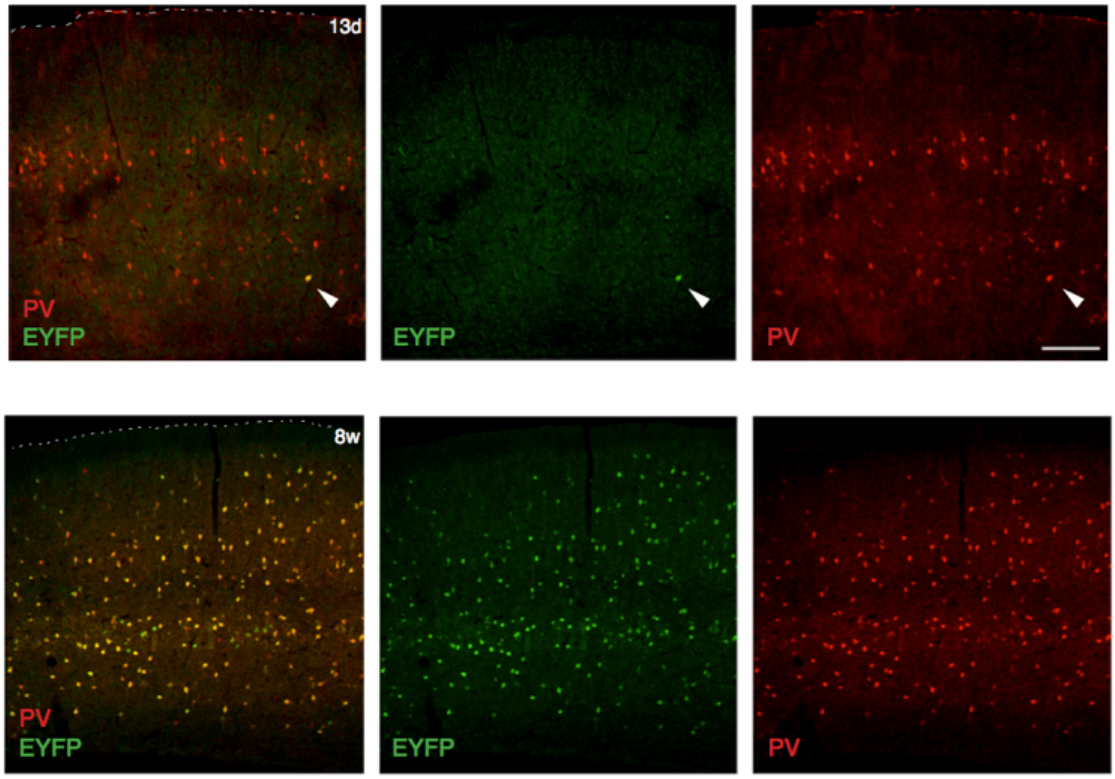
For viral transduction, 5-7 weeks old mice were anesthetized with an

intraperitoneal injection of a mixture of ketamine (1.1 mg/kg) and xylazine (0.16 mg/kg) and AAV DIO ChR2-mCherry was injected into hippocampus (1.8 mm posterior to Bregma and 1.5 mm from the midline; n = 5 each for PV-Cre and PV-Cre, NR1f/f mice). 7-10 days after viral transduction, transverse hippocampal slices (400 µm thick) were prepared in ice-cold dissection. Slices were incubated in an interface incubation chamber containing extracellular artificial cerebrospinal fluid (aCSF) gassed with 5 % $CO_2$ and 95 % $O_2$ (pH 7.4), allowed to recover for 30 minutes at 30° C and kept for another 30 minutes at room temperature. The slices were then transferred to a submerged recording chamber and continuously perfused with aCSF. The tungsten bipolar electrode (FHC) was placed in the stratum radiatum or oriens and the Schaffer collateral/commissural fibers were stimulated at 0.1 Hz. The patch recording pipettes (2-5 MΩ resistance) for whole-cell recording were filled with internal solution. Picrotoxin (0.15 mM, Sigma) was dissolved in aCSF to block $GABA_a$ receptor-mediated synaptic transmission for whole-cell patch clamp recordings. AMPA receptor-mediated EPSCs were recorded at -70 mV, and NMDA receptor-mediated EPSCs were recorded at +40 mV with the same stimulus strength in the presence of NBQX (0.005 mM, Tocris). All experiments were performed at room temperature. Experiments and the analysis of data were performed in a blind manner. Results are reported as mean ± SEM. Statistical significance was evaluated by Student-t test with statistical significance set at $P < 0.05$.

# 3. Results

## 3.1 General characterization of PV-Cre/ NR1f/f mice

**Fig. 3.1** Recombination in PV-Cre mice



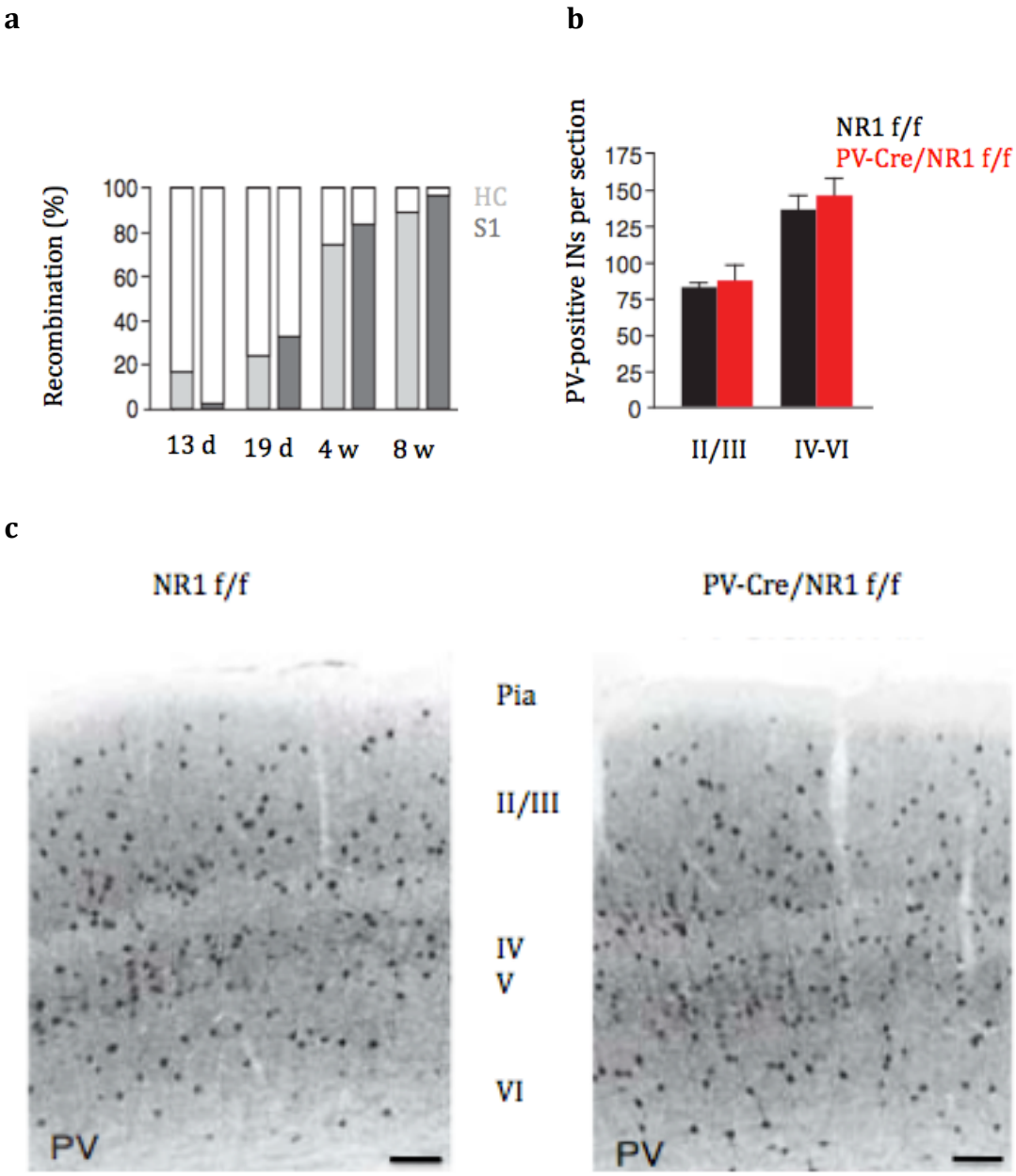

*Top: at P13 (13 days after birth), only scattered PV-interneurons have recombined in somatosensory cortex (white arrowhead).*
*Bottom: at 8w (8 weeks after birth) nearly complete recombination can be seen in somatosensory cortex of PV-Cre mice.*

*Scale bar: 200 µm.*

The conditional knockout mice (PV-Cre/ NR1f/f mice) developed normally and did not exhibit growth abnormalities as reflected in body weight or other gross anatomical changes. Cre-dependent recombination from the Parvalbumin locus followed the postnatal onset of Parvalbumin expression and was specific to PV-expressing interneurons, as shown in figure 3.1. PV-Cre recombination in

somatosensory cortex and in the hippocampus was initially detected at 13 days of age with increased recombination at 29 days and almost complete recombination at 8 weeks of age, as indicated above by co-localization of EYFP and PV in the reporter mouse line (see Fig. 3.1 and 3.2). For this quantification, brain sections of transgenic animals were immunohistochemically labeled for PV and within frames of sight, the number of marked cells was counted, as well as the number of genetically labeled cells. Microscopic images illustrate the result of this analysis.

**Fig. 3.2** Genetic ablation of NMDA-R specifically in PV-interneurons



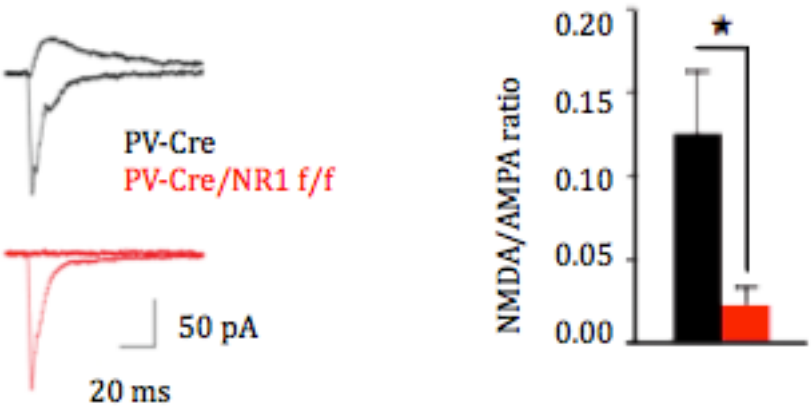
**a** *Quantification of recombination from the PV locus in somatosensory cortex and hippocampus at different time points. HC: Hippocampus. S1: Somatosensory cortex. INs: interneurons.*
**b** *Distribution of PV interneurons in NR1f/f and PV- Cre, NR1f/f mice, respectively, at 11 w in layer II/III and layer VI-VI. Error bars: mean ± SEM.*
**c** *Immunohistochemistry for parvalbumin interneurons in somatosensory cortex of adult NR1f/f and PV-Cre, NR1f/f mice.*

*Scale bar: 200 μm*

Intracellular recordings *in vitro* confirmed the functional loss of NMDA currents in fast spiking PV-expressing interneurons in PV-Cre/ NR1f/f mice (recorded from 5 cells in 4 PV-Cre/ NR1f/f mice and from 7 cells in 5 in PV-Cre (control) mice) from hippocampal slices (see Fig. 3.3). The cells used for recording were identified by the red fluorescent protein mCherry, which indicated that virus transduction after the injection had taken place and knockout of the receptor subunit could be expected.

**Fig. 3.3** Intracellular slice recordings

**a** **b**




**a** *Sample EPSC traces mediated by the AMPA-R (downward) and NMDA-R (upward) from a control PV-Cre mouse and a PV-Cre/NR1f/f mouse.*
**b** *NMDA-R EPSC/AMPA-R EPSC ratio in control and PV-Cre/NR1f/f mice. . *P < 0.05, error bars: mean ± SEM.*

In both groups of mice, we found the cortical architecture (layers and barrels in S1), migration and differentiation of PV-interneurons to be normal. Also, the total number of another interneuronal population, the somatostatin-expressing interneurons, was not changed in PV-Cre/ NR1f/f mice. The anatomy of other macroscopic brain structures like hippocampus, the ventricle system, striatum or the cerebellum, were normal, as could be evaluated in brain slices.

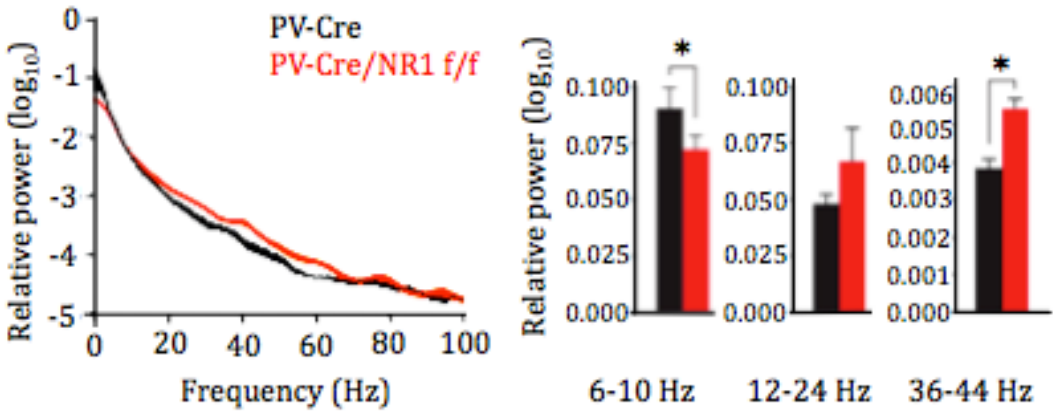
## 3.2 Spontaneous and induced brain rhythms

We have previously described that photoactivation of fast spiking PV interneurons after viral transduction for ChR2 enhances gamma oscillations in the rodent cortex (Cardin, Carlen et al. 2009). Following up these results, we recorded local field potentials in layers II/III and IV of primary somatosensory (barrel) cortex of anesthetized PV-Cre/ NR1f/f as well as in control mice (n = 8 sites in 5 controls and 10 sites in 6 PV-Cre/ NR1f/f mice) to identify potential changes in baseline cortical rhythms after depletion of the NMDA receptor on

PV-interneurons. As shown in fig. 3.4, we found a significant decrease in power in the alpha frequency band (6-10 Hz) and a significant increase in power in the gamma frequency band (36-44 Hz) compared to control animals.

**Fig. 3.4** Spontaneous LFP activity

**a** **b**




**a** *Relative power from 1-100 Hz in control and PV-Cre/NR1f/f mice.*
**b** *Relative LFP power in the alpha (6-10 Hz), beta (12-24 Hz) and gamma (36-44 Hz) frequency bands: in PV-Cre/ NR1f/f mice, relative LFP power in the alpha band (6-10 Hz) is decreased, while gamma activity (36-44 Hz) is increased. *P < 0.05. Error bars: mean ± SEM.*

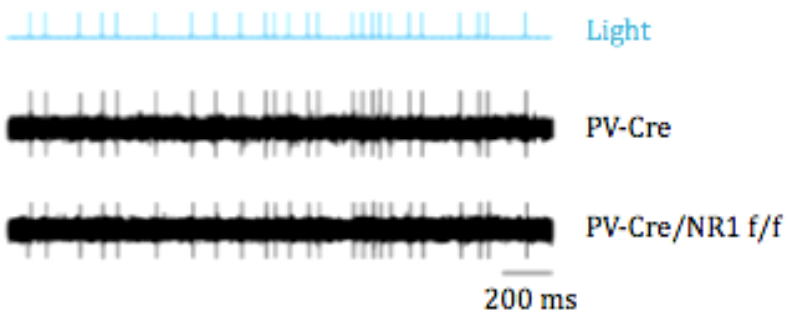
We next used optogenetic tools to examine the role of the NMDA-R in FS PV-interneurons for the induction of gamma oscillations in cortical networks. We directly activated FS PV-interneurons with and without functional NMDA receptors and compared the network responses.

We drove FS PV-cells with 1 ms laser-generated light pulses at a broad range of frequencies, from 8 to 200 Hz. Across all our samples, FS PV-interneurons in both control and NMDA-R-deficient mice were driven with high reliability by light pulses, although FS PV-interneurons lacking the NMDA receptor showed a significant increase in spike latency. As to be expected from our previous findings, light pulses in the gamma range (30-80 Hz) resulted in significant amplification of LFP power in control mice, shown in fig. 3.5. Activation of FS PV-interneurons in the conditional knockout mice also induced an

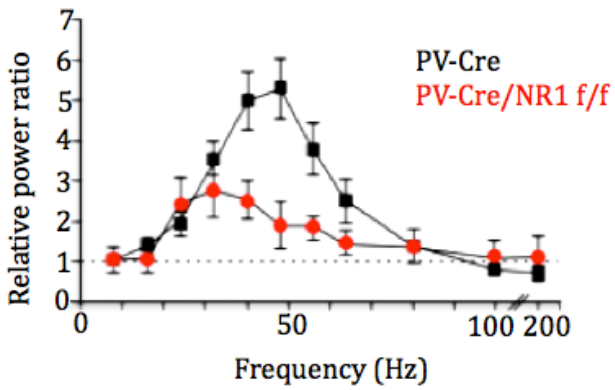
enhancement of gamma range activity in the local network ($P < 0.05$), but the induction of this enhanced activity was significantly lower than in control mice. Spontaneous LFP activity in slightly lower frequencies in the beta1 (15-19 Hz) or beta2 frequency band (20-30Hz) was not significantly altered in baseline conditions in PV-Cre/ NR1f/f mice.

**Fig. 3.5** Spontaneous and induced cortical gamma oscillations require NMDA-R in PV-interneurons

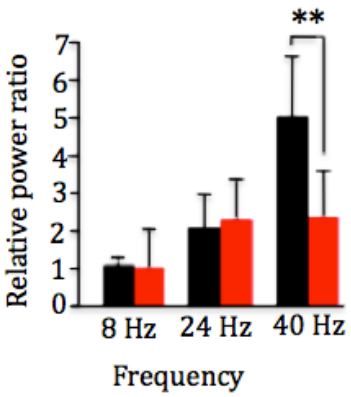
**a**




**b**




**c**



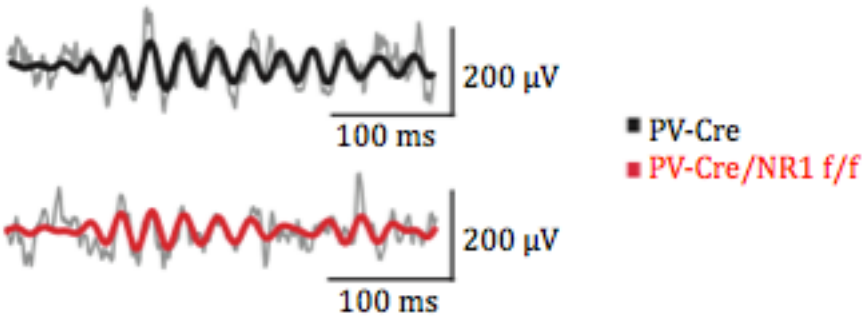

**a** *Photostimulation and single-unit recording from PV interneurons: middle trace from control mouse, lower trace from PV-Cre/NR1f/f mouse. A series of 1 ms light pulses (blue trace) was delivered in a random pattern drawn from a broadband distribution (5–200 Hz). In both cases, each light pulse evoked a single spike from the cell. Both cells followed the light stimulus with a high degree of reliability.*

**b** *Photoactivation of transduced FS PV-interneurons in the control and PV-Cre/ NR1f/f mice at 8, 24, and 40 Hz on relative LFP power in those frequency bands.*
**c** *Mean power ratio in each LFP frequency band in response to light activation of ChR2-expressing FS PV-interneurons at varying frequencies. PV-Cre/NR1f/f mice generate significantly less 30–60 Hz oscillations (gamma) than control mice. *P < 0.05, **P < 0.01. Error bars: mean ± SEM.*

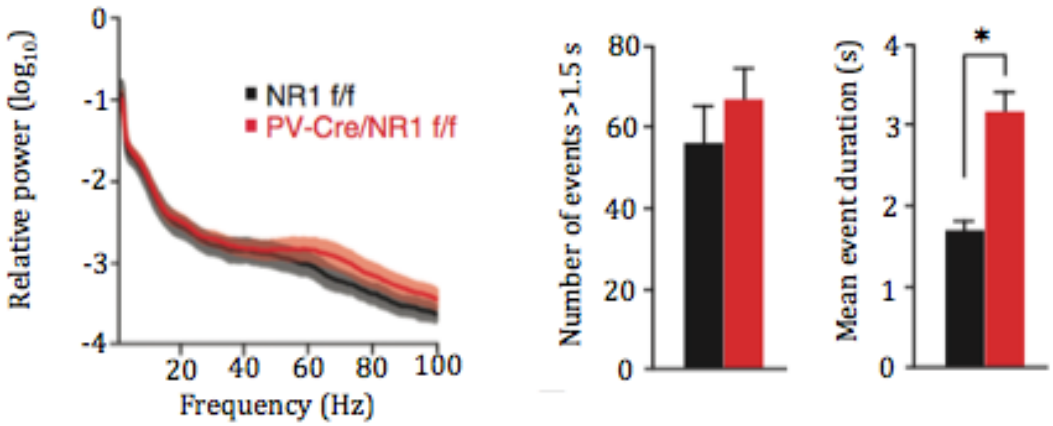
To test whether these deficits in cortical network activity were also detectable in awake behaving animals, we performed LFP recordings in layers II/III and IV of somatosensory cortex in freely moving PV-Cre/ NR1f/f and control mice (n = 7 for each group) during exploratory behavior. We detected prominent gamma band oscillations during exploration. We compared gamma rhythms during a baseline recording period and found that PV-Cre/ NR1f/f mice exhibited an elevation of spontaneous LFP in the gamma range that was non-significant and had significantly longer mean event durations for spontaneous gamma oscillations compared to control mice ($P < 0.005$), although the number of gamma events was unchanged.

**Fig. 3.6** Baseline cortical oscillations in S1 in awake behaving animals

**a**



**b** **c**



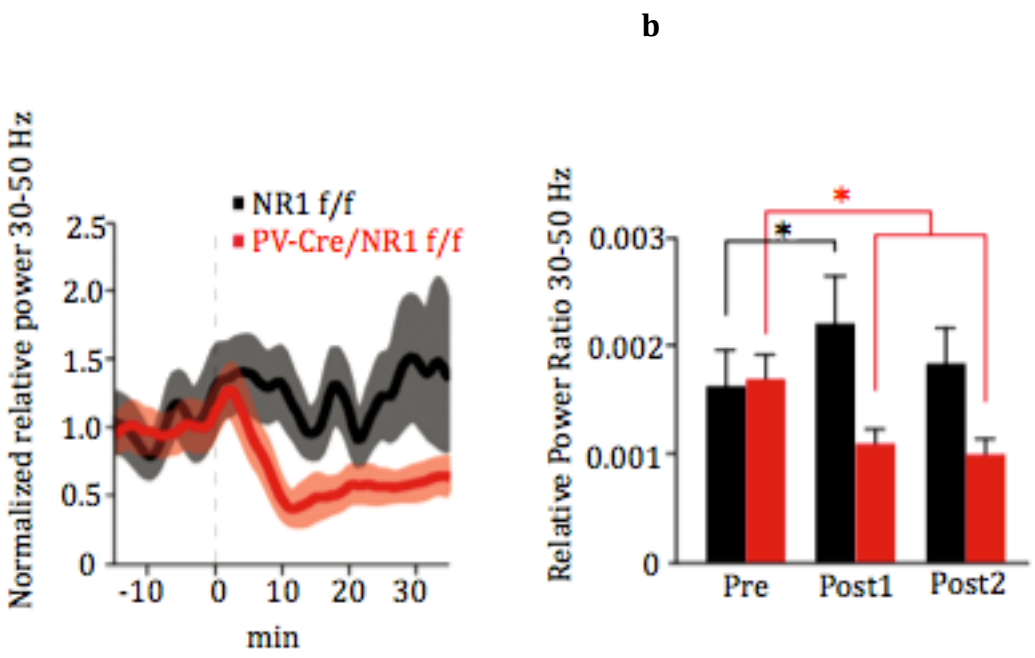

**a** *Examples of single-trial gamma activity from PV-Cre/NR1f/f and control mouse. Thin lines: LFP filtered between 5-300 Hz; thick lines: LFP filtered in the gamma frequency range (30-50 Hz).*
**b** *Mean power spectra 1–100 Hz. Lighter regions indicate SEM.*
**c** *Characteristics of gamma events (30-50 Hz). Mean event duration is significantly increased in freely moving PV-Cre/NR1f/f mice. *P < 0.05. Error bars: mean ± SEM.*

We next challenged PV-Cre/ NR1f/f mice with an acute administration of the non-competitive NMDA antagonist MK-801 (0.5 mg/kg IP) to investigate the contribution of NMDA-R in PV-interneurons to evoked gamma activity in the awake state. Control mice acutely displayed a significant increase in LFP in the gamma range (30-50 Hz) (Post1, 5-15 min after MK-801 administration; $P < 0.05$), which is the outcome that had been observed before by other groups (Ehrlichman, Gandal et al. 2009).

In contrast to this, PV-Cre/ NR1f/f mice displayed a significant reduction in gamma band activity after administration of the drug. The reduced gamma band activity after NMDA antagonist treatment in the conditional knockout mice supports the hypothesis that FS PV-interneurons are a main target of pharmacological NMDA blockade, which in turn is associated with altered gamma rhythms. Both groups displayed a significant induction in the alpha frequency band relative power at later time points (6-10 Hz; Post2, 25-35 min

after MK-801 administration).

**Fig. 3.7** Effects of NMDA-R antagonist MK-801 on neuronal population activity




**a** *Average power in the gamma frequency band (30-50 Hz) 15 min before administration of MK-801 (dashed line), until 35 min after administration of the drug. Lighter regions indicate SEM.*
**b** *Average power in gamma frequency band before and after administration of MK-801. Pharmacological treatment produces a significant increase in gamma power in control mice (black) and a significant reduction in gamma power in PV-Cre/ NR1f/f mice (red) (Post1 is 5-15 min after MK- 801 administration; Post2 represents 25-35 min after MK-801 administration).*
**P < 0.05. Error bars: mean ± SEM.*

The relative power in the 12– 24 Hz (beta frequency) band remained unchanged in control animals during the same time frame, but was significantly reduced in PV-Cre/ NR1f/f animals. Injections of saline, as another control experiment, did not induce any significant changes in locomotor behavior or relative power in any frequency band between genotypes throughout the duration of the recording sessions.

In summary, our results obtained through anesthetized and awake

electrophysiology have demonstrated that

1. cortical baseline gamma rhythms are disrupted when FS PV-interneurons lack NMDAR-dependent input

and

2. the ability to induce additional gamma oscillations in the network is significantly reduced in PV-Cre/ NR1f/f mice, which might be an indication of impaired network flexibility.

## 3.3 Behavioral Effects of NMDA-R Depletion in PV-Interneurons

The PV-Cre/ NR1f/f mice and their control littermates were further characterized in a series of paradigms to assess their general locomotor and exploratory behaviors, and also in cognitive tasks evaluating learning and memory.

### 3.3.1 General Activity

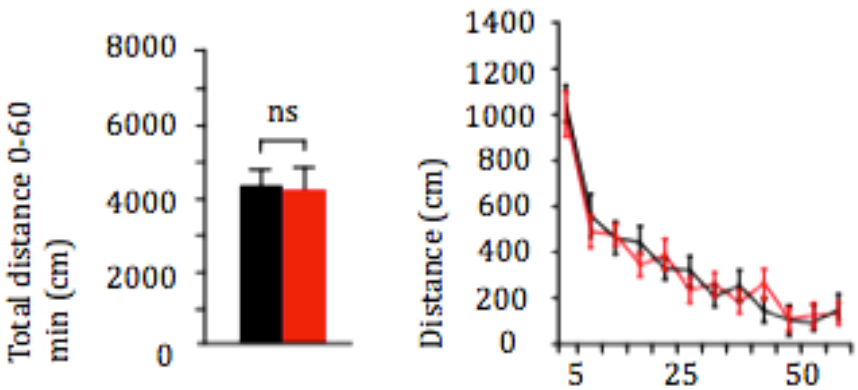
Mice were first introduced into an automated novel open field environment at the age of 7 weeks (n = 11 per genotype), which is the age when behavior tests are consensually done. It is important for these tests to have stable conditions, and some of these tests, like PPI, require functionality in systems that change at older ages. Hearing loss starts early in rodents, so animals that are supposed to be tested in paradigms relevant for neuroscientific research have to be mature enough to be fully developed, but also young enough to still yield the critical qualities. We thus started with the tests that require young age, followed by the remaining tests with breaks of at least 24 hours in between. However, we did not observe any consistent behavioral differences between PV-Cre/ NR1f/f and control mice over the entire 60-minute trial of the Open Field or in any 5

minute block after analysis of 18 different parameters, including total distance, locomotion, stereotypy and center vs. margin time.

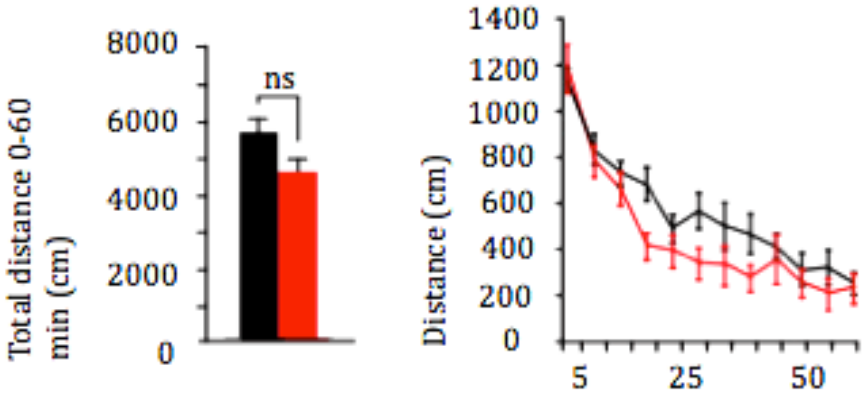
Because of this unexpected finding, we questioned whether PV-Cre/ NR1f/f mice at later stages might develop cognitive or behavioral abnormalities and therefore assessed mice of 11-12 weeks of age (n = 9 per genotype) in the same test as done before with younger animals. We found a small but significant difference in general activity, represented by the total distance travelled over the 60-minute period, with control mice exhibiting a larger increase in distance traveled at 11-12 weeks than at 7 weeks, compared to the difference in PV-Cre/ NR1f/f mice at 7 and 11-12 weeks. Analysis of time spent in the center or margin of the open field, vertical movements or stereotypy counts did not reveal differences between the genotypes over the 60-minute period, although there were some significant differences in individual blocks of 5 minutes. While these small age dependent effects may be of interest in light of behavioral changes associated with transitions from adolescence to adulthood, our results suggest a subtle behavioral effect of NMDA-R deficiency in PV-interneurons in the unchallenged state. This finding is in contrast to the phenotypes of hyperlocomotion and stereotypical behaviors in mice with general, pancortical NMDA hypofunction (Mohn, Gainetdinov et al. 1999).

**Fig. 3.8** PV-Cre/NR1f/f mice display discrete behavioral abnormalities

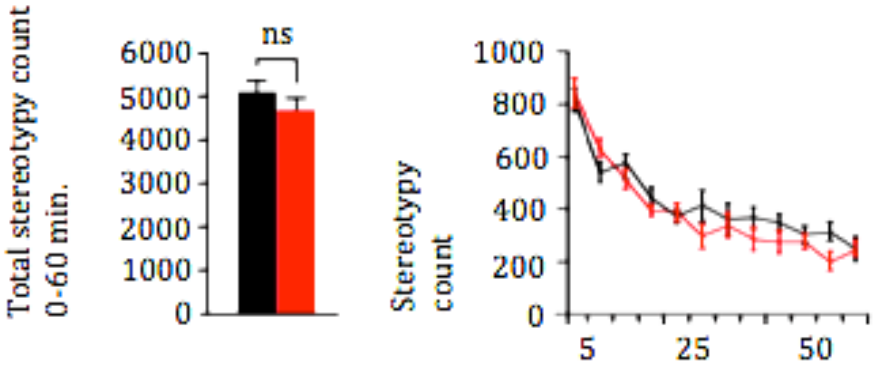
**a**



**b**




**c**



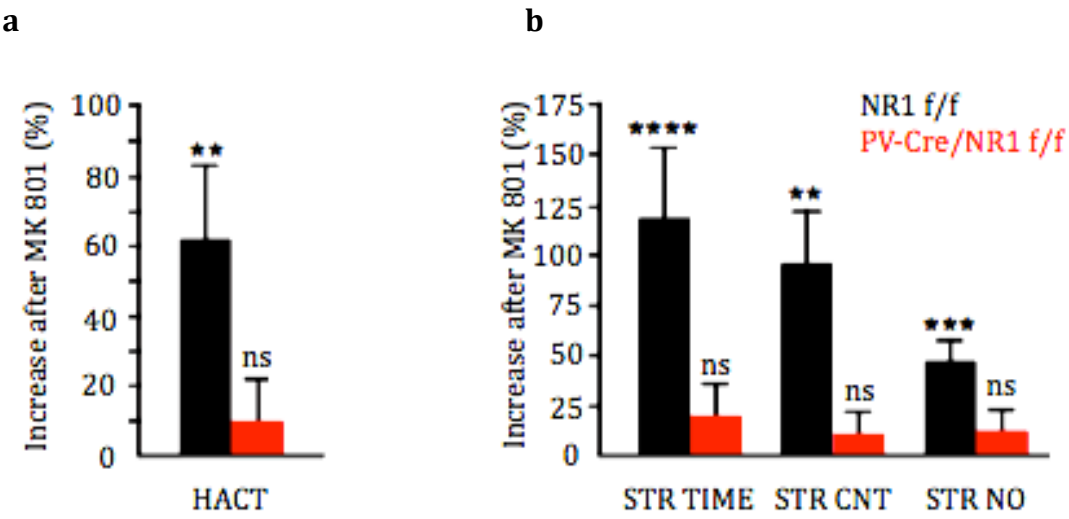

**a** *Total distance travelled in the open field of PV-Cre/NR1f/f and control mice at 7 w.*
**b** *Non-significantly increased total distance in control mice compared to 7 w and compared to PV-Cre/ NR1f/f mice at 11 w, respectively.*
**c** *PV-Cre/NR1f/f mice do not display stereotypy behavior. Error bars: mean ± SEM. Ns: non-significant.*

#### 3.3.1.1 Pharmacological Manipulation

Our electrophysiological findings indicate that the PV-Cre/ NR1f/f mice have a selective deficit in gamma emergence when FS PV-interneurons are optogenetically driven or when NMDA-Rs are pharmacologically inhibited. To investigate potential behavioral differences in response to acute NMDA-R blockage, we exposed naïve mice of both groups (n = 11 per genotype) to the open field for 30 minutes and watched the changes induced by administration of the NMDA antagonist MK-801 at a low dose (0.3 mg/kg) in the same

experimental setting for another block of 60 minutes. Control mice responded to MK-801 administration by increased horizontal activity and induction of stereotypies, as has been previously reported (Mohn, Gainetdinov et al. 1999). PV-Cre/ NR1f/f mice did not show similar behavioral responses to MK-801 administration, which again supports our hypothesis from electrophysiological findings that PV-interneurons are a main target of NMDA antagonists.

**Fig. 3.9** Effect of NMDA-R antagonist MK-801 on behavior in the open field test




**a** *MK-801 treatment induces significant increase in horizontal activity only in control mice (compared to behavior before administration of MK-801).*
**b** *MK- 801 treatment results in marked increase in stereotypy behavior only in control mice. ns: non-significant, *P < 0.05, **P < 0.01,* ***P < 0.001, ****P < 0.0001*. Error bars: mean ± SEM.*

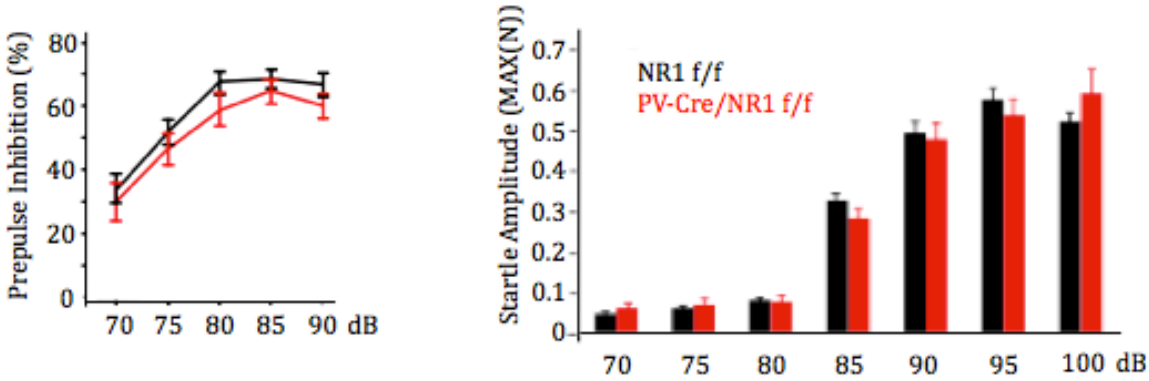
### 3.3.2 Prepulse Inhibition and the Acoustic Startle Reflex

As mentioned before, prepulse inhibition (PPI) abnormalities have been suggested to represent an endophenotype of schizophrenia, associated with deficiencies in sensory information filtering. Interneurons are considered to be of importance for phase-dependent inhibition and circuit oscillations that in turn regulate information processing. Our evaluation of PPI in PV-Cre/ NR1f/f mice in comparison to control mice did not reveal any significant changes in PPI over several prepulse intensities (70-100 dB) (n = 17 control mice and 15

PV-Cre/ NR1f/f mice). Critically, the startle response was also not significantly different between control mice and PV-Cre/ NR1f/f mice, which excludes motor or sensory defects as artifact.

**Fig. 3.10** PPI and ASR are normal in PV-Cre/NR1f/f mice

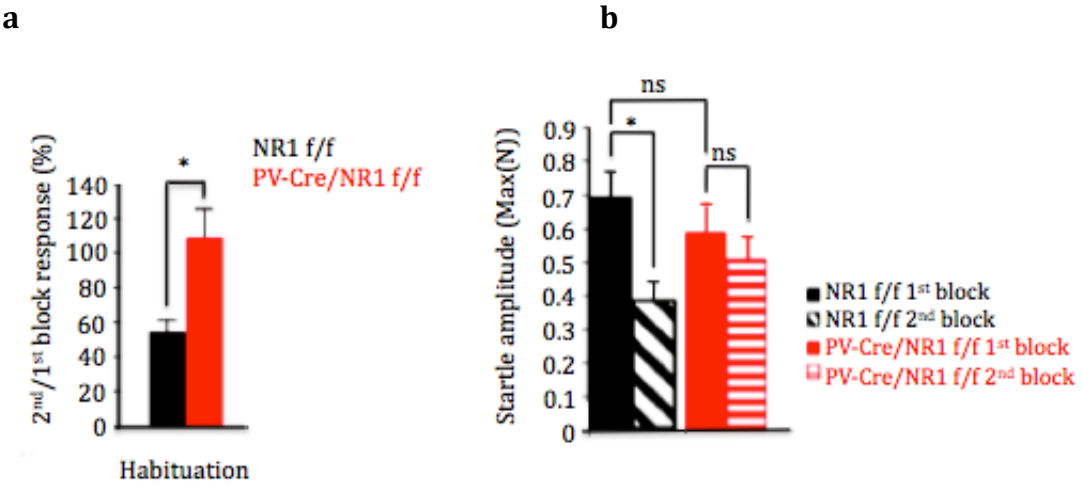
**a** **b.**




**a** *PV-Cre, NR1f/f mice do not show a deficiency in sensorimotor gating as measured in PPI.*
**b** *Startle response (Max(N)) from 70-120 dB in control and PV-Cre/ NR1f/f mice.*

We did, however, observe in PV-Cre/ NR1f/f mice a significant reduction in habituation between the two sets of single tones, which were presented in the beginning and at the end of a testing session.

**Fig. 3.11** PV-Cre/NR1f/f display deficiencies in habituation

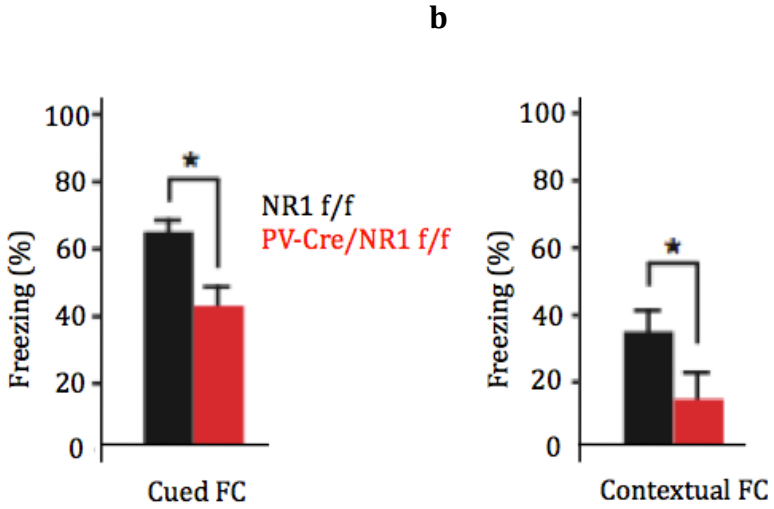



**a** *Ratio of Startle intensitiy between the first and the second block, in the beginning and in the end of the test.*
**b** *Control mice show almost 50% reduction in the intensity of the startle response while NMDA-R deficient mice do not habituate. ns:non-significant, *P < 0.05. Error bars: mean ± SEM.*

### 3.3.3 Learning and Memory tested in Fear Conditioning and Morris Water Maze

This finding of habituation deficits generated the need to further evaluate the memory function of PV-Cre/ NR1f/f mice. Therefore, we next tested both groups in established tests in the same category of learning and memory, the cued and contextual fear conditioning paradigms (n = 5 per genotype). Mice were exposed to a neutral conditioned stimulus (sound) in a novel environment, paired with an aversive foot shock as the unconditioned stimulus. When tested 24 h later, NMDA-R deficient mice exhibited reduced freezing behavior both in a tone-dependent trial ($P < 0.05$, Mann-Whitney test) and in a contextual version of the test ($P < 0.05$, Mann-Whitney test), which argues for the importance of NMDA-dependent recruitment of PV-expressing interneurons in the amygdala and in hippocampal circuits for proper memory formation.

**Fig. 3.12** PV-Cre/NR1f/f mice exhibit impaired freezing behavior both to a tone-dependent and a contextual version of fear conditioning

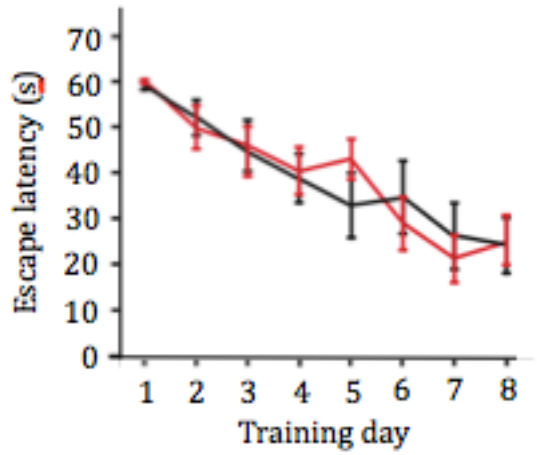



*Fear conditioning:*
**a** *Tone-dependant (cued) version.*
**b** *Contextual version. *P < 0.05; error bars: mean ± SEM. n = 5 per genotype.*

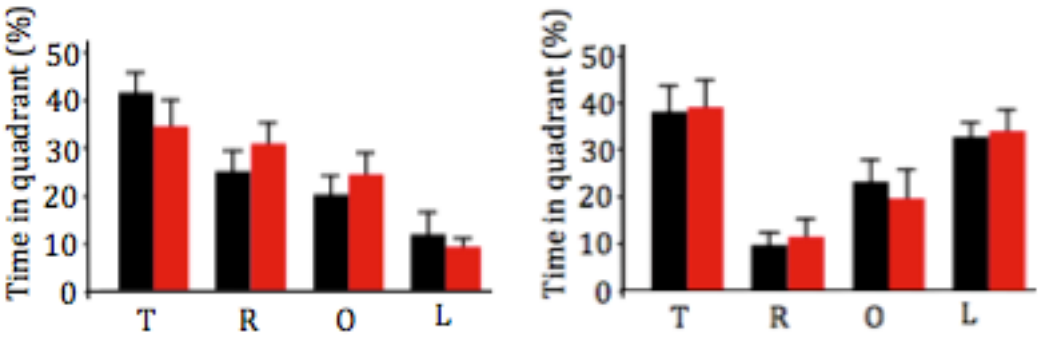
Learning and memory was further tested in a water maze task with a fixed hidden platform to evaluate spatial reference memory. Mice were tested in two trials per day with a 5 minute intratrial interval. The rate of learning in the water maze task over 8 days was not impaired in conditional knockout mice compared to controls (escape latency, $P = 0.8794$, two-way analysis of variance (ANOVA); n = 8 control and 11 PV- Cre/ NR1f/f mice). The probe trial on day 9 did not reveal significant differences in target quadrant preference compared with control mice ($P = 0.33$ for target quadrant, unpaired t-test). To also address reversal learning, we introduced the hidden platform in a different quadrant and continued the learning paradigm for 4 days. Learning rate of the new platform location over 4 days was not significantly different between genotypes ($P = 0.513$, two-way ANOVA) and a probe trial on the 5$^{th}$ day did not reveal target quadrant preference differences between genotypes ($P = 0.9$ for target quadrant, unpaired t-test). Thus, PV-Cre/ NR1f/f mice do not exhibit deficiencies in spatial memory formation or retrieval and apparently have the ability to reverse this learning using the same spatial cues.

**Fig. 3.13** Performance of both groups in the Morris Water Navigation Task

**a**




**b** **c**

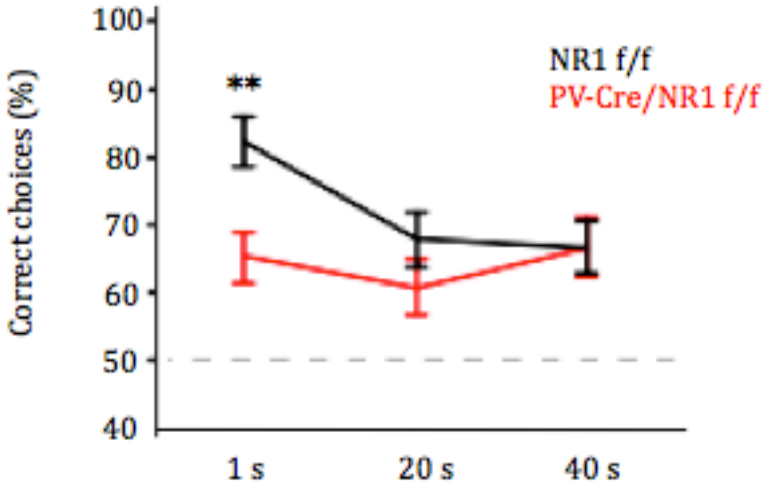



**a** *PV-Cre/NR1f/f perform similarly to control mice during training in a hidden platform version of water maze*
**b** *Time spent (%) in each quadrant during the water maze probe trial*
**c** *Time spent (%) in each quadrant during the water maze probe trial after reversal training.*
*T: target quadrant; R: right quadrant; O: opposite quadrant; L: left quadrant.*

Working memory, or operant memory, describes the normal function of animal and human brains to store and retrieve information from the past few seconds or minutes and make immediate decisions based on them. Deficits of schizophrenic patients (Goldman-Rakic 1999) in this system implicate NMDA-R as a critical component of working memory, with inhibition playing an important role in the process of shaping time-dependent firing of pyramidal cells in neocortical circuits during cognitive tasks (Constantinidis, Williams et

al. 2002). Patients with schizophrenia display aberrant gamma rhythm induction during working memory load; NMDA antagonists disrupt working memory in humans (Krystal, Karper et al. 1994) and also in rodents. We assessed working memory in a T-maze task, using a variant developed to reflect clinical assessment of patients (n = 10 control and 9 PV-Cre/ NR1f/f mice). Mice were first trained, then observed in 10 trials per mouse (n = 12 per genotype) each day with varying intratrial intervals to evaluate effects of working memory load on performance. PV-Cre/ NR1f/f mice performed similarly to control mice at 40-second intratrial intervals, and not significantly different at 20-second; only their performance at the 1-second intratrial interval revealed a remarkable difference between genotypes. In this category, our conditional knockout mice achieved 65% accuracy whereas control mice were significantly better with 82% accuracy ($P < 0.01$, unpaired t-test). This means that in the system we tested here, PV-Cre/ NR1f/f mice performed at similar accuracy levels independent of working memory load, in contrast to control mice, which start at higher accuracy levels at short intratrial intervals, but show decaying performance over longer intervals. This, in turn, implies that for short versus longer delays, different pathways might be used, and only the one for very short-term operations seems to be affected by depletion of the NMDA-R on PV-expressing interneurons.

**Fig. 3.14** Accuracy levels in the Discrete Paired-Trial Variable-Delay T-Maze Task




*Only an intratrial delay of 1 second reveals a difference between the genotypes. **P < 0.01; error bars: mean ± SEM.*

Electrophysiological findings and the results from behavior tests together characterize brain functions of mice lacking the NMDA-R on FS PV-interneurons. These mice have altered gamma rhythms and respond differently to pharmacological treatment with NMDA-R antagonist MK-801, in electrophysiological as well as in behavioral categories. Behavior tests did not reveal any phenotype of higher anxiety or show deficits in sensorimotor gating, also their performance in a test for spatial memory was not different from control mice. However, conditional knockout mice show clear deficits in learning and memory in several experimental tests, but not in all of them.

# 4. Discussion

## 4.1 Results from our Model

Our experiments showed altered circuit dynamics resulting from NMDA-R deficiency in FS PV-interneurons. Mice lacking the NR1 subunit of the NMDA-R in those cells show enhanced resting state gamma expression in LFP recordings and decreased induction of gamma frequency oscillations following optogenetic activation of virally transduced PV-interneurons. These findings resemble characteristic changes after low dose-administration of non-competitive NMDA-R antagonists and they are in accordance with data acquired from psychiatric patients, who display diminished evoked gamma rhythm in response to cognitive and sensory tasks (Basar-Eroglu, Brand et al. 2007). NMDA-R deficiency seems to particularly affect the gamma range frequency, and interestingly, the cortical network of PV-Cre/ NR1f/f mice is less flexible to induction of gamma rhythms by direct activation of FS PV-interneurons. The knockout mice, however, do not display behavioral features that are commonly associated with animal models of schizophrenia, but they also fail to react in a schizophrenia-like manner when treated with NMDA-R antagonists.

We found a phenotype of diminished habituation, which reflects learning deficits. This is a new finding since the particular role of NMDA-R on interneurons alone had not been investigated before in this context. Results from the T-maze task led to the conclusion that rapid initiation of the working-memory encoding phase is likely to require the NMDA-R on PV-interneurons, while the memory retention phase might be mediated primarily by dopamine-regulated circuits (see fig. 3.12, where the two groups start at significantly different levels, but the difference vanishes with longer delays).

## 4.2 A parallel Approach

In a very similar approach to what was described here, another group tested a conditional knockout mouse in electrophysiological, behavioral and histological aspects (Belforte, Zsiros et al. 2010). They generated two different mouse lines lacking the NR1 subunit of the NMDA-R on GABAergic interneurons, one with early postnatal onset, the other knockout effective in early adulthood. In both cases, Cre-expression was driven by Ppp1r2 gene promoter (protein phosphatase 1, regulatory (inhibitor) subunit 2). Immunohistochemical stainings revealed that in the early postnatal knockout, "40-50% of the cortical GABAergic interneurons across several cortical regions" were Cre positive and thus cells in which recombination and knockout presumably took place, 70% of those being Parvalbumin-positive and 15% positive for Neuropeptide Y (Belforte, Zsiros et al. 2010). In total, they argue to have knocked out NR1 in 70% of cortical PV-interneurons. Those details seem to be important because their findings mirror ours in some aspects but are strikingly different in others. In electrophysiological experiments, they also found asynchrony and hyperactivity of pyramidal neurons lacking GABAergic inhibition. In behavioral aspects, however, they found "features reminiscent of human schizophrenia", demonstrated in PPI deficits, abnormal social and nest-building activity and hyperlocomotion in the open field test (Belforte, Zsiros et al. 2010). When challenged with the NMDA-R-antagonist MK-801, those symptoms became visible in the control mice but did not change in the knockout group. Interestingly, in the cells lacking the NR1 subunit, levels of both GAD67 and of PV were diminished. Decreased expression of GABA, GAD67 and PV in postmortem brain tissue from schizophrenic patients had been reported before (Torrey, Barci et al. 2005), but the fact that dysfunctional NMDA receptors alone could lead to this biochemical change rises new questions. Does decreased glutamate signaling downregulate GAD67-expression? And, since there is a loss of Parvalbumin in postmortem tissue from patients, how far is Parvalbumin, often primarily regarded a marker for a class of interneurons, actually involved in intracellular and intercellular processes? Belforte et al. found the phenotype resembling schizophrenia in

only one of their knockout lines, the one with an early postnatal onset of the knockout. When NR1 is depleted at a later time point, those characteristics in focus (PPI deficits, hyperactivity and abnormal social behavior in the social recognition test) could not be detected, which might be the core difference to explain the different outcomes of their approach versus ours. In context of our finding it is also worth to ask, does the histochemical classification of interneurons represent clear functional or clinical relevance? We investigated only the selective conditional knockout of NR1 on one subpopulation of interneurons, while Belforte et al. did not make a distinction between different kinds of GABAergic interneurons. And yet, there is evidence that especially the Calcium-binding protein Parvalbumin might play a functional role in the generation of gamma oscillations. Sejnovski et al. just very recently published modeling results - a theoretical approach to the same questions asked here. Since Parvalbumin binds to Calcium, a second messenger involved with release of neurotransmitter from vesicles in the presynaptic terminal, a change in the intracellular concentration could interfere with synaptic coupling between interneurons and pyramidal cells and thus compromise network activity. In their computational model, they found that "PV-deficient GABA synapses had increased asynchronous release of GABA, which decreased the level of excitation and reduced gamma band activity" (Volman, Behrens et al. 2011).

## 4.3 Models for Schizophrenia

Yet another way to test for molecular patterns in schizophrenia was, among others, carried out by Lodge et al., who have argued in the same line of our hypothesis that the robust finding of decreased PV-expression in postmortem tissue of schizophrenic patients could give an explanation for the positive symptoms of schizophrenia. They used a common pharmacological model for schizophrenia, where prenatal MAM administration (Moore, Jentsch et al. 2006) produces a specific phenotype resembling schizophrenia and also a loss of PV-interneurons in several brain regions of rats (Lodge, Behrens et al. 2009). They detected a pathological loss of PV-interneurons throughout the medial PFC and ventral hippocampus, associated with altered prefrontal and

hippocampal activation during task performance in freely moving rats and thus reasoned that "a deficit in intrinsic GABAergic signaling may be the origin of the hippocampal hyperactivity purported to underlie the dopamine dysfunction in psychosis." Taking into consideration that clinical studies with schizophrenic patients have shown increased baseline activity in hippocampal regions (Malaspina, Corcoran et al. 2008) it is worthwhile to discuss the effects of changes in interneuronal capacities not only in cortical, but also limbic structures. Moreover, PV-interneurons are also present for example in the thalamus, especially in the medial nuclear group, and in the cerebellum. Thalamic inhibition plays an important role in sensory information processing and in synchronizing cortical activity, while the cerebellum and especially the cerebellar vermis seem to be crucial for the startle reflex (Cheng and Rühlmann, manuscript in final editing). This adds more complexity to the question of where the molecular and structural correlates for the psychopathology of schizophrenia might be found.

Neuroscience is challenged when models are needed for disruptions in some of the most important features of the human brain. Studying brain functions in context of psychiatric diseases that change the very basis of personality is a task that requires several steps of abstraction and interpretation. Furthermore, a skeptic mind would find that objectivity is impossible to maintain during the process of investigation, partly because any idea can only be investigated with the techniques at hand and read with the knowledge of the very moment. Current opinions reflect a collective understanding of the details in questions, but we might find assumptions that seem logical and useful today proven wrong at any time in the future. This seems to be true for models of disease, for general explanation of physiological phenomena and for diagnostic classification. A short excursion: Ketamine administration has been considered a good model for changes in a schizophrenic brain and was first described in detail by Krystal et al. in the early 1990s (Krystal, Karper et al. 1994). The most remarkable feature of this model is the induction of disorganization, which presents as thought disorder and behavioral disorganization, but interestingly it only rarely induces hallucinations. So it might be reasonable to think that this

group of symptoms, which happens to be the least understood of the three (positive, negative, and cognitive symptoms), is almost certainly not caused primarily by an imbalance of glutamatergic excitation of inhibitory interneurons. Also, the administration of ketamine in healthy humans does not affect reactions measured in PPI (Oranje, Gispen-de Wied et al. 2002) (see Oranje et al. Biol Psychiatry 2002). The unchanged responses in PPI of healthy individuals treated with ketamine, in contrast to decreased PPI in schizophrenic patients, has led scientists to believe that dopaminergic dysfunction underlies the altered function in sensorimotor gating. If that is the case, then why do Belforte et al. see a phenotype of disrupted sensorimotor gating, when neither pharmacological blockage of the NMDA-R induces this, nor our mouse model shows this specific type of behavior? How far do the network changes lead that are induced by NMDA-R depletion on inhibitory interneurons?

Krystal et al. even reported of a case (Corlett, D'Souza et al. 2010) where in a healthy individual under experimental settings, ketamine administration induced Capgras syndrome. This is striking, because Capras is a disorder normally caused by traumatic brain injury and shows features of psychosis. Patients do not hallucinate, but they suffer from what could be described as misperception of their own situation. In fact, Capgras patients think that their family members ore other close persons are being substituted by strangers, doppelgangers, in order to harm them in some way that does not have to be completely logical. They recognize their relatives' faces, but then somehow come to believe that those are not the people they pretend to be. What does this have to do with the projects and findings discussed here? It gives a hint to further think about the subgroups of schizophrenic symptoms, of the role of NMDA-R-antagonists, perception and consciousness.

Interestingly, another group of researchers just recently published clinical data from schizophrenic patients. Following the idea that NMDA-R encephalitis, a paraneoplastic syndrome mainly affecting young female persons, resembles schizophrenia very much, they took samples from patients diagnosed with

depression, bipolar disorder or schizophrenia and found antibodies of several classes against the NR1 subunit of the NMDA receptor in serum and CSF samples in 9.9% of schizophrenic patients (Steiner, Walter et al. 2013).

Schizophrenia, as mentioned in the introduction, is characterized by symptoms that appear as if they were several diseases within one diagnosis. Genetic research points in the same direction, and from clinical surveillance it seems to be the case that medications targeting specific neurotransmitter systems can offer remedy for one group of symptoms but at the same time worsen others.

Limitations of animal models lead back to genetic analysis of biological tissue from affected persons and to non-invasive techniques reflecting brain activity, anatomical changes and postmortem analyses. Animal models can serve well when biological assays answer our questions. But since we do not know if mice have consciousness, thoughts, any sense of reality and themselves in it, we cannot find out if a mutant mouse might show these core symptoms of psychiatric diseases. We can only invent better paradigms that investigate results of what we think is altered in specific pathologic conditions.

## 4.4 Conclusion

We have shown the important role of the NMDA-R on PV-interneurons on gamma rhythm induction and specific cognitive functions. Mice lacking a subunit of the NMDA-R only in Parvalbumin-positive interneurons develop normally and do not show obvious pathological changes, but a closer look reveals disrupted brain rhythms, measured in LFP recordings, and also the inability to respond to direct optogenetic activation of PV-interneurons with synchronous pyramidal cell firing in the gamma range frequency, in the way control mice do. Non-competitive NMDA-R antagonists produce symptoms of schizophrenia and also disrupt synchrony of neuronal activity. We have previously shown that PV-interneurons modulate gamma activity in the rodent

cortex, and with this project we have also proven that it is glutamatergic signaling onto GABAergic interneurons that governs their synchrony, which seems to be necessary for cognitive functions in both animals and humans. Our conditional knockout mice performed normal, compared to the control group, in behavioral tests for sensorimotor gating, anxiety and spatial memory. They did, however, show a phenotype of altered habituation and learning in three different test paradigms. Interestingly, the T-maze test showed a difference between groups in short delay-paradigms, but not in the longer ones. This gives rise to the thought that the formation of working memory and its retrieval depend on circuits and neurotransmitter systems that change with the details of the task. When control mice were treated with the non-competitive NMDA-R antagonist MK-801, they showed a pattern of altered brain activity that has been studied excessively before. They also performed differently in behavioral tasks, showing increased stereotypic movements and general activity in the open field test, while mice with deficient NMDA-Rs on PV-interneurons do not show any of these reactions. This supports our hypothesis that interneurons are the main effective target of psychoactive drugs antagonizing the NMDA-R.

# 5. Summary

We investigated a conditional knockout mouse line lacking the NR1 subunit of the NMDA receptor on Parvalbumin-expressing (PV-) interneurons in electrophysiological, behavioral and neurobiological paradigms. Our hypothesis that dysfunction of the NMDA-R on PV-interneurons contributes to disruption of brain rhythms and such disruption could be causing symptoms of schizophrenia was based on several observations. PV-interneurons had previously been shown to modulate synchronous firing of pyramidal cells in the γ-frequency, putatively through a cycle of GABAergic inhibition. Schizophrenic patients undergo changes in the gamma rhythm and PV-interneurons orchestrate gamma oscillations. Further, there is evidence that NMDA-Rs play an important role in schizophrenia. Ketamine, a non-competitive NMDA-R antagonist, has for years been used as a model for schizophrenia research because it induces symptoms of schizophrenia in healthy individuals and worsens symptoms in schizophrenic patients. It also leads to desynchronization of pyramidal cell activity and worsens performance in memory tasks. Moreover, Ketamine produces general cortical activation in fMRI studies in humans. These two lines of evidence pointed into the direction of the NMDA-R on PV-interneurons being an important factor in the etiology of schizophrenia.

The role of PV-interneurons in gamma rhythm induction was characterized in a previous project of our group, using a PV-Cre mouse and optogenetic means. We extended this result by crossing the PV-Cre mouse line with a strain of mice carrying floxed alleles for NR1. This resulted in a conditional knockout of that gene that began at the age of approximately 8 weeks. After viral injections into Barrel Cortex, the site of following LFP recordings, Cre-expressing PV-interneurons expressed ChR2, an ion channel selective for positively charged ions, which can be activated by blue light delivered by a laser. This tool enabled us to activate the specific cell population of virally transduced inhibitory interneurons and measure the electrical field activity following this activation.

Glutamatergic pyramidal cells receive input from various cortical and subcortical regions and they feed an inhibitory pathway by activating interneurons, which in turn project onto the same group of pyramidal cells. It appears that the intensity of this inhibition from interneurons takes a wave-like shape and determines the frequency of pyramidal cell firing as they respond to input from cortical and subcortical structures. We were particularly interested in the generation and disruption of gamma range oscillations of around 40 Hz. These oscillations appear crucial for cognitive processes and altered gamma rhythms have been reported as a consistent trait of schizophrenic individuals. Baseline gamma activity is elevated in patients suffering from schizophrenia, but the amplitude of gamma events evoked by mental tasks does not reach the relative level of healthy persons.

Unlike a study that used a closely related mouse model, we did not find a phenotype of hyperlocomotion, anxiety or disrupted sensorimotor gating – all of which are considered characteristics equivalent to symptoms of schizophrenia. Still, mice lacking the NMDA-R on PV-interneurons revealed a pattern of brain waves similar to that of schizophrenic patients; a finding that has now been reproduced by several groups. Knockout mice failed to respond with appropriate elevation of gamma amplitude when PV-interneurons were directly activated by optogenetic stimulation. In our recordings in layers II/III and IV of primary somatosensory cortex, conditional knockout mice also showed a significant decrease in power in the alpha frequency band and a significant increase in power in the gamma frequency band compared to control animals. The typical electrophysiological and behavioral changes induced by NMDA-R antagonists observed in control mice were not induced in the knockout mice. The effect of psychoactive drugs mimicking symptoms of schizophrenia could thus be communicated by NMDA-Rs on inhibitory interneurons. We also found that knockout mice performed significantly worse than control mice in tasks that require short-term memory and basic learning abilities.

Habituation to a sensory stimulus is seen in humans and rodents and interpreted as a primitive learning ability that allows adaptation to the environment. We found a significant habituation deficit in conditional knockout mice. In addition, we used the T-Maze task, which was developed to test working memory and to study mechanisms of generating and retrieving memories in a short period of time. Knockout mice performed significantly worse than control mice at the shortest intratrial delay, but showed no difference in longer delays between the first (forced) and the second (choice) run in the maze. This implies that the rapid initiation of the memory-encoding phase could depend on the NMDA-Rs, but that a longer delay activates different networks, which in our mouse model were either not affected or had been compensated for. The memory tasks that are affected in our mouse model seem to require gamma oscillations, while long-term learning tasks like the water maze do not depend on this fast network activity.

Our findings support the idea that NMDA-Rs on PV-interneurons are a key factor in generating gamma oscillations. They potentially also play a role in the symptoms of schizophrenia, if this can be concluded within the limitations of animal models for psychiatric diseases. We measured altered neuronal activity in selected cortical regions of knockout mice and we also found impairments in memory and learning, which could be correlating features. However, it is unclear which underlying factors (genetic mutations, environmental changes, misbalance in neurotransmitter systems after drug abuse) lead to uncompensated neurobiological impairments in humans that produce the symptoms of schizophrenia. It is possible that a chain of events causes one symptom. Also, an imbalance of glutamate- and GABA-signaling is very likely to result in changes in dopamine-mediated brain functions. We found another piece in the puzzle that is research in the field of schizophrenia, where (epi-) genetic, molecular, pharmacological, environmental and biographic factors seem to play a role in the etiology and progression of the disease.

# 6. Deutsche Zusammenfassung

In der hier beschriebenen Arbeit wurde untersucht, welche Auswirkungen zu beobachten sind, wenn die durch NMDA-Rezeptoren vermittelte Aktivierung inhibitorischer Interneurone des Gehirns aufgehoben ist. Die Rolle der Interneurone ist besonders im Hinblick auf die Generierung von γ-Oszillationen von Bedeutung. Es handelt sich hierbei um synchrone neuronale Aktivität von 20-80 Hz, welche anscheinend die Voraussetzung für kognitive Leistungen und Aufmerksamkeit sowie für die Integration sensorischer Stimuli bildet. Laut gängigen Hypothesen könnte eine Störung der neuronalen Synchronizität an der Pathophysiologie der Schizophrenie beteiligt sein. Somit stellt das vorgestellte Projekt auch einen Versuch dar, molekulare Charakteristika der Schizophrenie zu finden.

Die Annahme, dass zwischen NMDA-R-vermittelter Aktivität inhibitorischer Interneurone, γ-Oszillationen und Symptomen der Schizophrenie ein kausaler Zusammenhang besteht, entstand aus verschiedenen Beobachtungen. NMDA-R-Antagonisten wie Ketamin, MK-801 und PCP verursachen in niedrigen Dosierungen schizophreniforme Zustände bei gesunden Probanden. In fMRI-Studien wurde sogar gezeigt, dass Ketamin zu einer Steigerung kortikaler Grundaktivität führt. Darüber hinaus verstärken die genannten Substanzen die Symptome von Schizophreniekranken. In vergleichenden EEG-Studien wurden veränderte Muster von γ-Oszillationen bei schizophrenen Patienten gefunden.
Die Beobachtungen unserer Arbeitsgruppe, die dieser Arbeit vorausgingen, bestätigten die Annahme, dass γ-Oszillationen nur durch die Interaktion zwischen inhibitorischen und exzitatorischen Neuronen möglich sind. Kortikale Pyramidenzellen erhalten Informationen von verschiedenen kortikalen und subkortikalen Strukturen, welche sie, in Abhängigkeit von ihrem Grundzustand, integrieren und weiterleiten können. Dieselben Pyramidenzellen aktivieren über den Neurotransmitter Glutamat inhibitorische Interneurone, welche die Pyramidenzellen in der Folge durch

Ausschüttung von GABA rekurrent hemmen. Die Intensität dieser Inhibition scheint wellenartig zu- und wieder abzunehmen. Dementsprechend werden Signale, welche die Pyramidenzellen erreichen, nur dann fortgeleitet, wenn die Inhibition schwach genug und das ankommende Signal stark genug ist. In der Literatur wird diese Theorie als „gamma cycle“ beschrieben.

Es lässt sich also postulieren, dass eine Aufhebung der inhibitorischen Wirkung von Interneuronen und die daraus resultierende Desynchronisation neuronaler Aktivität die zugrunde liegende Ursache der oben beschriebenen Effekte von NMDA-R-Antagonisten sein könnte. Um diese Hypothese zu überprüfen, wurde ein Mausmodell entwickelt, welches als „conditional knockout“ dysfunktionale NMDA-Rezeptoren auf Parvalbumin-exprimierenden (PV-) Interneuronen trägt. Dieses Mausmodell wurde im Hinblick auf elektrophysiologische Eigenschaften charakterisiert und in unterschiedlichen Verhaltenstests untersucht. Außerdem wurden die Interneurone selektiv mittels optogenetischer Verfahren *in vivo* aktiviert und die Effekte dieser Aktivierung ebenfalls elektrophysiologisch in LFP-Messungen untersucht.

Die Optogenetik ist ein relativ neues Instrument, das in der Neurowissenschaft für ganz unterschiedliche Fragestellungen benutzt wird. Ein virales Konstrukt wurde in stereotaktischen Operationen in die kortikalen Bereiche injiziert, in denen später elektrophysiologische Experimente durchgeführt wurden. In Cre-exprimierenden Zellen führte die so eingebrachte DNS zu Rekombination und Expression von Channelrhodopsin2(ChR2)-mCherry. ChR2 ist ein lichtaktivierbarer Ionenkanal, der auf Licht einer bestimmten Wellenlänge mit einer Konformationsänderung reagiert und durch den Einstrom von Kationen zu einer Depolarisation der vorher transduzierten Neurone führt. Auf diese Weise ist eine spezifische und kontrollierte Aktivierung von Populationen und Klassen von Nervenzellen mittels blauen Lichts *in vivo* möglich.

Die Methode des „conditional knockout“ bietet mehrere Vorteile gegenüber einem konventionellen Knockout, bei dem eine Mutation schon in Keimbahnzellen implementiert wird. Um diese spezielle Form des Knockouts

zu erhalten, wurden PV-Cre Mäuse mit einer Linie gekreuzt, die „gefloxte“ NR1-Untereinheiten des NMDA-Rezeptors besitzt. Das bedeutet, dass der Genabschnitt, der für diese bestimmte Untereinheit des NMDA-Rezeptors kodiert, auf beiden Seiten von einer palindromischen Sequenz flankiert wird, welche der Cre-Rekombinase als Erkennungspunkte dienen. Sobald der Promoter, unter dem die Cre-Rekombinase steht, in diesem Fall also PV, exprimiert wird, wird der „gefloxte“ Genabschnitt herausgeschnitten. Ein Gen wird auf diese Weise zelltypspezifisch und zu einem bekannten postnatalen Zeitpunkt entfernt.

Die Aktivität der Cre-Rekombinase in PV-Interneuronen wurde im vorliegenden Projekt einerseits für den „conditional knockout“ des NMDA-Rezeptors benötigt, führte aber zusätzlich in Kombination mit Virusinjektionen zur zelltypspezifischen Expression von ChR2. Die NMDA-R-depletierten Interneurone exprimierten durch Transduktion nach Virusinjektionen den lichtaktivierbaren Ionenkanal ChR2. Diese spezielle Population von Interneuronen konnte somit selektiv aktiviert werden und es konnten die Auswirkungen des dysfunktionalen NMDA-Rezeptors auf generelle Netzwerkeigenschaften untersucht werden.

Die Regulation von Synchronizität und Frequenz neuronaler Aktivität ist von enormer Bedeutung. Geordnete Aktivität, also synchrone oder korrelierte elektrische Potenziale von Populationen von Nervenzellen, bildet die Grundlage für höhere kognitive Funktionen. Sie stellt gleichzeitig eine potenzielle Schwachstelle für das Entstehen von neurologischen und psychiatrischen Krankheiten dar. Anscheinend ist bei Personen, die unter Schizophrenie leiden, die im EEG und MEG messbare Grundaktivität in der schnellen γ-Frequenz höher als bei Gesunden. Bei der Erfüllung von Denkaufgaben bleibt jedoch die Steigerung der γ-Oszillationen aus, die man bei Gesunden beobachtet. Ein ganz ähnliches Muster wurde bei der Untersuchung des beschriebenen Mausmodells gefunden. Im Grundzustand war höhere γ-Aktivität messbar als bei Kontrollmäusen. Die direkte optogenetische

Aktivierung von PV-Interneuronen führte nicht zu einer Erhöhung der γ-Aktivität, was bei Kontrollmäusen zuverlässig gelang.

Die Auswertung von Verhaltenstest der selektiv NMDA-R-depletierten Mäuse ergab keinen Hinweis auf typische Veränderungen, die im Tiermodell als Äquivalente zu Symptomen von Schizophrenie gelten. Dazu gehören Hyperaktivität, stereotype Bewegungsmuster und Defizite im PPI-Test. Die generelle motorische Aktivität der Versuchstiere war jedoch im „open field test“ nicht gesteigert. Es gab keine Anzeichen für Stereotypien und es waren auch keine PPI-Defizite messbar. Mäuse, denen die NMDA-R-vermittelte Inhibition von exzitatorischen Pyramidenzellen fehlte, zeigten allerdings signifikante Defizite in verschiedenen Lerntest: Ihnen fehlte die Fähigkeit der Habituation an sensorische Stimuli, außerdem erbrachten sie verminderte Leistungen in Aufgaben, die als Tests für das Kurzzeit- oder Arbeitsgedächtnis angesehen werden können. Wie schon beschrieben, führen Ketamin und MK-801 bei gesunden Probanden zu Symptomen, die der Schizophrenie ähneln. Bei Mäusen führt die Applikation von NMDA-R-Antagonisten üblicherweise zu Veränderungen von Verhaltensweisen, z.B. induzieren sie Hyperaktivität und Stereotypien. Diese Veränderungen konnten bei Kontrollmäusen beobachtet werden, jedoch nicht im NMDA-R-depletierten Mausmodell. Insgesamt liefern die beschriebenen Ergebnisse starke Indizien dafür, dass der schizophrenieähnliche Effekt der NMDA-R-Antagonisten tatsächlich über den NMDA-Rezeptor auf Interneuronen vermittelt wird. Die Details der Verhaltenstests sind auch in Abgrenzung zu ähnlichen Mausmodellen bemerkenswert, in denen statt nur auf einer Population, der NMDA-R auf allen Klassen von Interneuronen durch „conditional knockout“ entfernt wurde. Durch welche Veränderungen die Symptome der Schizophrenie jedoch ursächlich entstehen, bleibt unklar. Anscheinend stellen Mutationen an verschiedenen Genloci jeweils eine Prädisposition für die Entwicklung psychiatrischer Erkrankungen dar. Der Pathophysiologie liegt wahrscheinlich ein Ungleichgewicht der elektrochemischen Vorgänge neuronaler Kommunikation zugrunde. Dieses Ungleichgewicht kann zwar plausibel, aber nicht vollkommen hinreichend durch Veränderungen in Teilen der

Neurotransmittersysteme erklärt werden. Hier ist fortwährende Forschungsarbeit nötig, um die Entstehungsmechanismen der Krankheit besser zu verstehen.

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München, im Mai 2014

# Curriculum vitae

## Persönliches

| | |
|---|---|
| Geburtsdatum | 19. Mai 1986 |
| Geburtsort | Hamburg |
| Vater | Dr. med. Bernd Rühlmann |
| Mutter | Rita Rühlmann |

## Forschung/Promotion

| | |
|---|---|
| Seit April 2013 | Postdoctoral Research Scholar in Neuroscience bei Prof. Kleinfeld an der **University of California, San Diego** (UCSD)<br><br>Forschungsgebiet: Neurovaskuläre Kopplung, *in vivo* 2-Photonen Mikroskopie und Elektrophysiologie |
| 2009 – 2011 | Mitglied in der Arbeitsgruppe von Prof. Konnerth am **Institut für Neurowissenschaften der TU München**.<br><br>Publikation: „Making waves: Initiation and propagation of cortico-thalamic $Ca^{2+}$ waves *in vivo*". Neuron 2013 |
| 2008 – 2009 | Experimentelle medizinische Doktorarbeit am **Picower Institute for Memory and Learning** des Massachusetts Institute of Technology (MIT) bei Prof. Li-Huei Tsai<br><br>Publikation: „A critical role for NMDA receptors in parvalbumin interneurons for gamma rhythm induction and behavior". Mol Psychiatry 2012 |

**Medizinstudium**

| | |
|---|---|
| 2012 | Staatsexamen – zweiter Abschnitt der Ärztlichen Prüfung |
| 2011 – 2012 | Praktisches Jahr an der TU München, Universität Zürich und Tel Aviv University mit dem Wahlfach Neurologie |
| 2007 – 2012 | Klinische Ausbildung an der TU München |
| 2007 | Physikum – erster Abschnitt der Ärztlichen Prüfung |
| 2005 – 2007 | Vorklinische Ausbildung an der LMU München |

Schulbildung

| | |
|---|---|
| 2005 | Abitur |
| 2002-2005 | Bilinguales Gymnasium des Schulzentrums an der Bördestraße, Bremen |
| 1996-2002 | Bilinguales Gymnasium des Schulzentrums an der Lerchenstraße, Bremen |
| 1992-1996 | Grundschule Vizelinstraße, Hamburg |