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**Patient-derived anti-NMDAR antibody disinhibits cortical neuronal networks
through dysfunction of inhibitory neuron output**

Abbreviated Title: anti-NMDAR antibody disinhibits cortical networks

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1. ABSTRACT

Anti-NMDAR encephalitis is a severe neuropsychiatric disorder associated with autoantibodies against NMDA receptors, which cause a variety of symptoms from prominent psychiatric and cognitive manifestations to seizures and autonomic instability. Previous studies mainly focused on hippocampal effects of these autoantibodies, helping to explain mechanistic causes for cognitive impairment. However, antibodies' effects on higher cortical network function, where they could contribute to psychosis and/or seizures, have not until now been explored in detail. Here, we employed a patient-derived monoclonal antibody targeting the NR1 subunit of NMDAR and tested its effects on *in vitro* cultures of rodent cortical neurons, using imaging and electrophysiological techniques. We report that this hNR1 antibody drives cortical networks to a hyper-excitable state and disrupts mechanisms stabilizing network activity such as Npas4 signaling. Network hyperactivity is in part a result of a reduced synaptic output of inhibitory neurons, as indicated by a decreased inhibitory drive and levels of pre-synaptic inhibitory proteins, specifically in inhibitory-to-excitatory neuron synapses. Importantly, on a single-cell level hNR1 antibody selectively impairs NMDAR-mediated currents and synaptic transmission of cortical inhibitory neurons, yet has no effect on excitatory neurons, which contrasts with its effects on hippocampal neurons. Together, these findings provide a novel, cortex-specific mechanism of antibody-induced neuronal hyper-excitability, highlighting regional specificity underlying the pathology of autoimmune encephalitis.

Key words: autoimmune encephalitis; NMDAR; autoantibodies; network excitability; cortical interneurons

2. SIGNIFICANCE STATEMENT

It is increasingly appreciated that the inadvertent activation of the immune system within CNS can underlie pathogenesis of neuropsychiatric disorders. Although the exact mechanisms remain elusive, autoantibodies derived from patients with autoimmune encephalitis pose a unique tool to study pathogenesis of neuropsychiatric states. Our analysis reveals that autoantibody against the NMDA receptor has a distinct mechanism of action in the cortex, where it impairs function of inhibitory neurons leading to increased cortical network excitability, in contrast to previously described hippocampal synaptic mechanisms of information encoding, highlighting brain regional specificity. Notably, similar mechanism of NMDAR-mediated inhibitory hypofunction leading to cortical disinhibition has been suggested to underlie pathology of schizophrenia, hence our data provide new evidence for common mechanisms underlying neuropsychiatric disorders.

3. INTRODUCTION

Over the last decade, a growing number of central nervous system disorders have been linked to autoantibodies, which have been detected in patients' cerebrospinal fluid (CSF). Many of these bind synaptic and neuronal cell-surface proteins, including a variety of neuro-transmitter receptors (Dalmau and Graus 2018). One of the most prevalent forms of autoimmune encephalitis is associated with IgG antibodies that bind N-methyl-D-aspartate receptors (NMDARs), a disease that is characterized by a rapid clinical progression and a broad range of symptoms (Dalmau et al. 2007). Most patients present with prominent psychiatric manifestations, including psychosis, hallucinations and behavioral changes, which further progress to severe memory loss, seizures and autonomic instability. Often patients require prolonged treatment in intensive care units (Irani et al. 2010). Interestingly, most are responsive to immunotherapies and show marked recovery (Titulaer et al. 2013). A fundamental question is how such antibodies mechanistically trigger such a broad range of symptoms in patients.

With regard to NMDAR encephalitis, most studies have focused on how autoantibodies cause memory deficits, in particular within hippocampal circuits. A framework for such investigations is based on a long-history of research showing that NMDARs are ligand-gated ion channels responsible for synaptic integration and plasticity and underlie hippocampal learning and memory processes (Bliss and Collingridge 1993; Nicoll 2017). Studies with patients' CSF reveal that most NMDAR-autoantibodies recognize epitopes within the extracellular domain of the obligatory NR1 subunit of NMDARs (Gleichman et al. 2012). Antibody binding primarily triggers the reversible internalization of NMDARs and thereby a net decrease in surface receptor clusters in hippocampal neurons (Hughes et al. 2010; Planagumà et al. 2015). Loss of receptors has been shown to decrease synaptic NMDAR-mediated currents (Hughes et al. 2010; Kreye et al. 2016) and to compromise synaptic plasticity (Mikasova et al. 2012; Q. Zhang et al. 2012; Würdemann et al. 2016). These conditions are thought to

115 contribute to memory deficits described in patients with NMDAR encephalitis and animal models
116 (Planagumà et al. 2016; Malviya et al. 2017).

117 At present, how such antibodies could also trigger psychiatric symptoms and/or epileptic seizures is
118 less clear, though actions at higher network levels, for instance within cortical circuits, seem likely.
119 Intriguingly, seizures have been observed in ~60% of encephalitis patients (Bruijn et al. 2019),
120 suggesting an increased excitability within these circuits (Manto et al. 2010). Yet there are few clues
121 of how NMDARs autoantibodies could drive this cortical hyper-excitability. On the other hand, it has
122 long been suggested that NMDARs are involved in the pathology of psychosis (“glutamatergic
123 hypothesis of schizophrenia”), wherein NMDAR antagonists such as ketamine and phencyclidine
124 (PCP) have been found to induce psychotic symptoms, as well as exacerbate them in schizophrenic
125 patients (Krystal et al. 1994; Jentsch and Roth 1999). This has led to the hypothesis that NMDAR
126 hypofunction, in particular on inhibitory interneurons (Belforte et al. 2010), could also cause
127 excitation/inhibition imbalances and disinhibition of prefrontal cortical circuits (Homayoun and
128 Moghaddam 2007a), similar to those observed in schizophrenic patients (Uhlhaas and Singer 2010).
129 It is currently unclear, whether auto-NMDAR antibodies could similarly affect cortical interneurons
130 and thus alter cortical network properties in patients with NMDAR encephalitis.

131 In the present study, we have evaluated whether a patient-derived antibody against the NR1 subunit
132 of NMDARs has direct effects on cortical neurons. Our detailed imaging and electrophysiological
133 analysis of cultured rodent cortical neurons revealed that this antibody increases network spiking
134 and bursting, driving cortical neurons into a hyper-active state. Surprisingly, this hyperactivity seems
135 to be a result of selective hypofunction of inhibitory interneuron output, specifically in inhibitory-to-
136 excitatory neuron synapses, leading to a decrease in inhibitory drive and hyper-excitability of these
137 cortical neuronal networks.

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4. MATERIALS AND METHODS

Preparation of cultured cortical neurons

All procedures for experiments involving animals were approved by the animal welfare committee of Charité Medical University and the Berlin state government. To distinguish inhibitory and excitatory neurons in some experiments (Fig. 4-8), we used GAD67-GFP (Δ neo)/+ (GAD67-GFP) mice, in which inhibitory neurons expressing GAD67 are fluorescently labeled (Tamamaki et al. 2003). In comparison to WT neurons, no changes in the electrophysiological properties of these GFP expressing neurons have been reported (Chang et al. 2014). To prepare primary cortical neuronal cultures for immunocytochemistry and mass culture patch clamp experiments, mouse WT (C57BL/6J) (RRID:IMSR_JAX:000664)(license: T0036/14) or GAD67-GFP animals (license: T0220/09) of either sex were prepared and grown on glass coverslips using the 'Banker Protocol' (Banker and Goslin 1988; Meberg and Miller 2003). In brief, astrocytes were prepared from mouse WT cortices on postnatal day zero to two (P0–2) and seeded on 6-well or 12-well plates at a density of 10,000 per 1 cm², 7-11 days before the addition of neurons. Cortical neurons were prepared from cortices dissected from mice P0–2 brains in cold HBSS (Millipore), followed by a 45 min incubation in enzyme solution containing DMEM (Invitrogen, Thermo Fisher Scientific), cysteine (3.3 mM), CaCl₂ (2 mM), EDTA (1mM), and papain (20 U/ml, Worthington) at 37°C. Next, the papain reaction was inhibited by incubating cortical tissue in a DMEM solution containing 10% FCS (Thermo Fisher Scientific), BSA (38 mM, Sigma-Aldrich), and trypsin inhibitor (95 mM) (Sigma-Aldrich) for 5 min. Afterwards, cells were triturated in Neurobasal-A medium (2% B27, 1% Glutamax, 0.2% P/S; Thermo Fisher Scientific) by gentle pipetting up and down. Isolated neuronal cells were plated onto nitric acid washed and poly-L-lysine-coated glass coverslips with paraffin dots, at a density of 50,000-200,000 per 12-well coverslip. After 1.5 h, the coverslips were placed upside down onto a bed of astrocytes and co-cultured in Neurobasal-A medium at 37°C, 5% CO₂, for 14–18 DIV before starting experiments.

163 To create autaptic cultures for single-cell electrophysiological recordings, cortical or hippocampal
 164 neurons were plated on micro-islands of astrocyte feeder layers, generated 1 week before the
 165 neuronal culture preparations, as described previously (Arancillo et al. 2013). Neurons were plated at
 166 a lower density (4,000 cells per 6 well coverslip) to obtain a single neuron on an astrocytic island.

167 For calcium imaging experiments, primary mouse cortical neurons were plated on top of a 1-week
 168 old astrocyte feeder layer, on round dishes containing a cell location grid at the bottom (μ -Dish 35
 169 mm, high Grid-500, Ibidi), at the density of 250,000 cells per dish. The cells were cultured in medium
 170 containing minimal essential medium (MEM; Sigma-Aldrich), insulin (25 mg/L, Sigma-
 171 Aldrich), glucose (20 mM, Sigma-Aldrich), L-glutamine (2 mM, Sigma-Aldrich), Pen/Strep (0.2%,
 172 Sigma-Aldrich), and NuSerum (10%, Becton Dickinson Labware). Seven days after plating, one-half of
 173 the culture medium was replaced with feeding medium as above, but lacking NuSerum and
 174 containing 0.5 mM of L-glutamine and 2% B-27 supplement (Invitrogen). Approximately one-half of
 175 the medium was then replaced with the feeding medium one or two times per week.

176 For multi-electrode array experiments, primary cultures of rat cortical neurons were prepared as
 177 described previously (Minerbi et al. 2009), using a protocol approved by the Technion committee for
 178 the supervision of animal experiments (IL-116-08-71). Briefly, cortices of newborn P1 Wistar rats
 179 (either sex; Charles River Laboratories) were dissected, dissociated by trypsin treatment followed by
 180 trituration using a siliconized Pasteur pipette. A total of $1\text{--}1.5 \times 10^6$ cells were then plated on thin-
 181 glass multielectrode array (MEA) dishes (Multi Channel Systems MCS), pre-coated with
 182 polyethylenimine (Sigma-Aldrich) for better cell adhesion. Subsequently, neurons were cultured at
 183 37°C and 5% CO₂, and grown in medium containing minimal essential medium (MEM; Sigma-Aldrich),
 184 insulin (25 mg/L, Sigma-Aldrich), glucose (20 mM, Sigma-Aldrich), L-glutamine (2 mM, Sigma-Aldrich),
 185 gentamycin sulfate (5 mg/ml, Sigma-Aldrich), and 10% NuSerum (Becton Dickinson Labware). At 7
 186 DIV, one-half of the culture medium was replaced with feeding medium lacking NuSerum and

187 containing 0.5 mM of L-glutamine and 2% B-27 supplement (Invitrogen) and then the cultures were
 188 fed in this way 3 - 4 times per week.

189 **Lentivirus production**

190 All lentiviral particles were provided by the Viral Core Facility of the Charite-Universitätsmedizin
 191 Berlin (<https://vcf.charite.de/en/>) and were prepared as described previously (Lois et al. 2002).
 192 Briefly, HEK293T (RRID:CVCL_0063) cells were cotransfected with 10 µg of shuttle vector, 5 µg of
 193 helper plasmid pCMVdr8.9, and 5 µg of pVSV.G with X-tremeGENE 9 DNA transfection reagent
 194 (Roche Diagnostics). Virus-containing cell culture supernatant was collected after 72 h and filtered
 195 for purification. Aliquots were flash-frozen in liquid nitrogen and stored at -80°C. Neurons were
 196 infected with lentivirus at 2–4 DIV.

197 **Production of monoclonal antibodies**

198 The human monoclonal antibody #003-102 is reactive to the NR1 subunit of NMDA receptors and
 199 was isolated from cerebrospinal fluid of a patient with acute NMDAR encephalitis (Kreye et al. 2016).
 200 Isotype-matched control antibody mG053 was isolated from blood of a healthy donor (Wardemann
 201 et al. 2003). For the recombinant expression of these antibodies, the paired expression vectors
 202 encoding for the antibodies' heavy and light chain were transiently transfected in HEK293T cells and
 203 purified from cell culture supernatants, as previously described (Kreye et al. 2016). The antibody
 204 concentration was determined using an anti-human IgG ELISA following the manufacturer's
 205 instructions (3850-1AD-6, Mabtech).

206 **Immunocytochemistry of cultured neurons**

207 *Npas4 experiment.* To verify the effects of the hNR1 antibody on somatic Npas4 expression, cortical
 208 neurons 14-17 DIV were incubated with 1 µg/ml of hNR1 antibody for 2h, 4h or 24h. Alternatively, to
 209 induce expression of Npas4, neurons were treated with NMDA (2 µM, Tocris) for 2h. Untreated cells
 210 were used as a control. To study the effect of hNR1 and NMDAR antagonist (2R)-amino-5-

211 phosphonovaleric acid (AP5) on NMDA-induced Npas4 expression, NMDA treatment was preceded
 212 by 6h or 24h of hNR1 (1 μ g/ml) or 3h of AP5 (100 μ M, Tocris) treatment. After treatment, cells were
 213 fixed with 4% PFA in PBS for 10 min and washed 3x 5 min with PBS. Subsequently, cells were
 214 permeabilized and blocked with a solution containing 5% normal goat serum, 2% BSA and 0.1%
 215 Triton in PBS, for 1 h. Neurons were then incubated with primary antibodies against Npas4 (1:1500,
 216 rabbit, Activity Signaling) and MAP2 (1:2000, chicken, Millipore, AB5543) in antibody solution
 217 containing 2% BSA in PBS for 2h at RT. Cells were then washed 3x 5 min with PBS and incubated with
 218 the differently labeled secondary antibodies (1:1000 in antibody solution, Invitrogen, Thermo Fisher
 219 Scientific), for 2h at RT and washed 3x 5 min with PBS. Finally, coverslips were dipped in H₂O and
 220 mounted in Pro-Long Diamon Antifade Mountant (Thermo Fisher Scientific).

221 *Synaptic/extrasynaptic hNR1 labeling.* Cortical neurons of GAD67-GFP animals were subjected to live
 222 staining with hNR1 and anti-human secondary antibody. Briefly, neurons were incubated with
 223 1 μ g/ml of hNR1 in original culture NBA medium (see above) for 20 min at 10°C. Cells were then
 224 washed once with same medium and incubated with Alexa-568 conjugated anti-human secondary
 225 antibody (1:1000, Thermo Fisher Scientific) for 20 min at 10°C. Afterwards, neurons were again
 226 washed once with culture medium, fixed with 4% PFA, blocked, stained with antibodies against
 227 excitatory presynaptic vesicle protein VGLUT1 (1:4000, SySy 135 304, Synaptic Systems) or the
 228 inhibitory postsynaptic scaffold protein gephyrin (1:500, SySy 147 011, Synaptic Systems) and labeled
 229 with secondary antibodies and mounted as described earlier (see Npas4 experiment).

230 *Inhibitory pre-synaptic proteins labeling.* Cortical neurons from GAD67-GFP animals were incubated
 231 with 1 μ g/ml of hNR1 antibody in original culture NBA medium for 0h, 6h or 24h at 37°C.
 232 Subsequently, cells were fixed with 4% PFA, blocked, stained with antibodies against MAP2 (1:2000,
 233 AB5543, Millipore), GAD65 (1:750, MAB5406, Millipore), or VGAT (1:4000, SySy 131 003, Synaptic
 234 Systems) and labeled with secondary antibodies before mounting as described above.

235 **Image acquisition and quantification**

236 Immunocytochemical staining for Npas4 experiments was acquired on a Nikon Spinning Disk
 237 Confocal CSU-X microscope equipped with an air 20× Plan Apo objective lens (NA = 0.8), controlled
 238 via NIS-Elements software (Nikon). Images were then processed using ImageJ (RRID: SCR_003070).
 239 ROIs were manually drawn around cell somas. After background subtraction, the mean intensity
 240 values of the ROIs were measured and normalized to the untreated control.

241 hNR1 labeling and staining for inhibitory pre-synaptic markers were analyzed using a spinning disc
 242 confocal microscope (Carl Zeiss Axio Observer.Z1 with Andor spinning disc and cobalt, omnicron, i-
 243 beam laser) (Carl Zeiss, Andor) using a 63x (1.4 NA) or 100x (1.4 NA) Plan-Apochromat oil objectives
 244 and an iXon ultra (Andor) camera controlled by iQ software (RRID: SCR_014461) (Andor). Only
 245 dendrites in proximity to the soma were imaged, and inhibitory and excitatory dendrites were
 246 distinguished based on GFP signal. Images were processed using ImageJ (RRID: SCR_003070) and
 247 OpenView software (written by Prof. dr. Noam Ziv, Technion Institute, Haifa, Israel). In brief, for
 248 synaptic protein levels at GAD65 and VGAT puncta along dendrites were detected with set
 249 parameters: 6x6 pixels boxes were placed over puncta and the mean fluorescence intensities were
 250 measured, followed by subtraction of background values. For hNR1 labeling within excitatory
 251 synapses, hNR1-positive puncta were detected in a similar manner and then VGLUT1 intensities
 252 within hNR1-positive puncta were measured. A value above a threshold of 5x background intensity
 253 was considered VGLUT1-positive and thus synaptic. The remaining hNR1 puncta were considered
 254 extra-synaptic. In a similar manner, boxes around detected gephyrin puncta served to measure hNR1
 255 intensity for localization of hNR1 puncta to inhibitory synapses. Z-plane images were analyzed using
 256 Volume Viewer plugin in ImageJ. Values of all puncta per region of interest (individual dendrites)
 257 were averaged and considered one data point.

258 **Multi-electrode arrays**

259 Neuronal network activity was recorded continuously from MEA electrodes (59 electrodes, 30 μm
 260 diameter, arranged in an 8 × 8 array), as described previously (L. Hazan and Ziv 2020). Briefly, cells

grown on MEA dishes were continuously perfused with fresh feeding medium at a rate of 4 ml/day using an ultra-low-flow peristaltic pump (Instech Laboratories), and maintained at 37°C and 5% CO₂. After setting up the perfusion system, network activity was allowed to equilibrate for 1h and then 15h of baseline activity was recorded. Next, 1 µg/ml of hNR1 antibody, control antibody or 1 µM AP5 was manually pipetted into the MEA dish and added to the perfusion system. Cell spiking was recorded in the presence of the antibodies or 1 µM AP5 for 24h. Finally, saturating concentrations of AP5 (50 µM) were added to the dish to block remaining NMDARs, and the activity of the culture was recorded for additional 6h. Recordings from MEA dishes were performed using a commercial 60-channel headstage (inverted MEA-1060-BC, MCS) with a gain of 53× and frequency limits of 0.02–8500 Hz. This signal was further filtered with frequency limits of 150–3000 Hz and amplified (20×) using a filter/amplifier (FA60S-BC, MCS). Data acquisition was performed using custom software [Closed Loop Experiment Manager (CLEM)] (H. Hazan and Ziv 2017). Data were collected at 16 k Samples/s. Action potentials were identified as negative threshold-crossing events, with the threshold calculated as 5× root-mean-square of traces recorded at the beginning of each experiment. Data were imported, converted and analyzed for spiking and bursting activity using custom scripts in MATLAB (MathWorks). Data from each MEA dish was normalized to the activity of last 3 hours of baseline recordings. To test if there is a significant increase in spiking/bursting rate due to 24h treatment, for each network the average rate at the end of baseline (1h) was compared to end of 24h treatment (1h) by paired t-test. To quantify percentage increase in spiking/bursting rates at the beginning/end of respective treatments (24h treatment with antibodies or 1 µM AP5, 50 µM AP5 or recovery at the end of experiment), the spike/burst rate of 10 min intervals was measured.

Patch clamp electrophysiology

Whole-cell patch-clamp recordings were performed on mass or autaptic, cortical or hippocampal neurons at 14–18 DIV, after 24h incubation with 1µg/ml of hNR1 or control antibody (mGo53) (Wardemann et al. 2003). Cortical neurons from GAD67-eGFP mouse line were used to distinguish

286 inhibitory (GFP-positive) and excitatory (GFP-negative) cells. All recordings were obtained at ~25°C
 287 from neurons clamped at -70 mV with a Multiclamp 700B amplifier (Molecular Devices) under the
 288 control of Clampex 10.4 software (Molecular Devices). Data were sampled at 10 kHz and low-pass
 289 Bessel filtered at 3 kHz and series resistance was typically under 15 MΩ and compensated at 70%.
 290 During all recordings, except for chemically induced NMDA and evoked synaptic NMDA responses,
 291 neurons were immersed in standard extracellular solution consisting of 140 mM NaCl, 2.4 mM KCl,
 292 10 mM HEPES, 10 mM glucose, 2 mM CaCl₂, and 4 mM MgCl₂. Chemically induced whole-cell NMDA
 293 responses were measured in extracellular solution containing 0 mM Mg²⁺, 0.2 mM CaCl₂, and 10 μM
 294 glycine, whereas synaptically evoked NMDAR currents were measured in extracellular solution
 295 containing 0 mM Mg²⁺, 2mM CaCl₂, and 10 μM glycine. The borosilicate glass pipettes (3–8 MΩ) were
 296 pulled with a micropipette puller device (Sutter Instruments) and filled with the internal solution
 297 containing 136 mM KCl, 17.8 mM HEPES, 1 mM EGTA, 0.6 mM MgCl₂, 4mM ATP-Mg, 0.3 mM GTP-Na,
 298 12 mM phosphocreatine, and 50 U/ml phosphocreatine kinase. All solutions were adjusted to pH 7.4
 299 and osmolarity of ~300 mOsm.

300 To selectively induce NMDA, GABA and kainate currents, NMDA (10 μM, Tocris), GABA (5 μM, Tocris),
 301 or kainic acid (20 μM, Tocris), were acutely applied to the neurons for 1 second by use of a fast-flow
 302 system. For synaptic responses in autaptic cultures, excitatory postsynaptic currents (EPSCs) were
 303 evoked by brief somatic depolarization of neurons from -70 mV to 0 mV for 2 ms. Synaptic NMDAR
 304 currents were measured in the presence of AMPAR antagonist NBQX (10 μM, Tocris).

305 All mass culture recordings were performed in the presence of TTX (0.5 μM, Tocris) to block
 306 propagation of action potentials. To identify spontaneous mEPSCs and mIPSCs, neurons were
 307 additionally immersed in AP5 (50 μM) and bicuculline (20 μM, Tocris), or AP5 (50 μM) and NBQX (10
 308 μM), respectively. Traces were recorded at a holding potential of -70 mV and were filtered at 1 kHz,
 309 mEPSCs and mIPSCs were detected by a template algorithm in Axograph X (Axograph Scientific).
 310 False-positive events were excluded by subtracting events detected from traces in the presence of

311 NBQX (10 μ M) or bicuculline (20 μ M) for mEPSCs and mIPSCs, respectively. To identify excitatory
 312 neurons for detection of mIPSCs in mass cultures (Fig. 4B-F), WT mouse neurons were infected with
 313 pLenti_CamKIIa_mKate2, a lentivirus expressing mKate2 under a CamKII promoter, at 3-4 DIV. For
 314 experiments detecting mEPSCs (Fig. 2E-F), GAD67-GFP line neurons were used.

315 Electrophysiological data were analyzed offline using Axograph X (RRID:SCR_014284) (Axograph
 316 Scientific), Excel (Microsoft), and Prism (GraphPad).

317 **Calcium imaging**

318 Cortical cultures grown on grid-bottomed dishes were infected with f(syn)-NES-jRCamP1b-WPRE-w, a
 319 lentivirus expressing jRCamP1b under the Synapsin promoter (Viral Core Facility, Charité –
 320 Universitätsmedizin), at 4 DIV. Images were acquired at 37°C and a CO₂ controlled environment, using
 321 Nikon Spinning Disk Confocal CSU-X microscope with an air 20× Plan Apo objective lens (NA = 0.8),
 322 controlled via the NIS-Elements software (Nikon). Time-lapse images were collected at 5 Hz using an
 323 iXon3 DU-888 Ultra camera (Andor) and 561 nm excitation laser. In each dish, 3 fields of view with
 324 approximately 10-20 cells were selected, and 2 min of spontaneous activity was measured twice,
 325 with 5 min interval between the runs. To study bicuculline-induced changes in network activity,
 326 bicuculline (30 μ M) was manually pipetted into the cell culture dish immediately after the end of the
 327 second baseline activity recording. After a 30 second waiting period, two 2-minutes imaging sessions
 328 were recorded, separated by a 5 min waiting interval.

329 Time-lapse image analysis and automatic time-series event detection were accomplished with a
 330 custom-written script in OpenView software (Prof. dr. Noam Ziv, Technion, Israel). In brief, ROIs were
 331 manually selected by placing boxes of 27 x 27 pixels over visually identified neuronal cell somas. Only
 332 active cells were included in analysis. Time series fluorescence values were converted into $\Delta F/F$ by
 333 calculating the ratio between the change in fluorescence signal intensity (ΔF) and baseline

334 fluorescence (F0). The custom-written algorithm identified the timestamps of calcium transient
 335 onset, which were then averaged per min to obtain the frequency of events.

336 **Experimental design and statistical analysis**

337 GraphPad Prism was used to analyze and represent data. Schematic figures were created with
 338 BioRender.com. Statistical design, sample sizes, and tests for each experiment can be found in the
 339 figure legends. All figures represent data from at least 3 independent experiments (independent
 340 cultures). Unpaired and paired t-tests and ANOVA Tukey's multiple-comparison tests were used to
 341 evaluate statistical significance.

342

343 **5. RESULTS**

344 **hNR1 antibody increases spiking and bursting of cortical networks**

345 Previous studies have shown that human anti-NMDAR antibodies affect NMDAR function in
 346 hippocampal neurons, helping to explain potential mechanistic causes for cognitive impairment in
 347 patients with auto-immune NMDAR encephalitis (Hughes et al. 2010; Mikasova et al. 2012;
 348 Würdemann et al. 2016). However, other symptoms centered in cortical circuits, such as psychiatric
 349 manifestations, catatonia, coma and epilepsy, have yet to be explored. We were thus interested in
 350 addressing the question of whether these antibodies can also affect cortical network function and if
 351 so, what is their functional impact? To explore these fundamental issues, we took advantage of a
 352 patient-derived monoclonal antibody #003-102 targeting the NR1 subunit of NMDAR (hNR1) (Kreye
 353 et al. 2016) and tested its effect on primary cortical neuron cultures. We began with examining
 354 effects of hNR1 antibody on neuronal network activity by growing cortical neurons on multi-
 355 electrode array (MEA) dishes for 11-14 days. This approach allows a continuous and simultaneous
 356 monitoring of activity from dozens of neurons recorded by 59 electrodes. Initially, baseline spiking
 357 activity of the neuronal network was recorded for 15h, afterwards 1 µg/ml of hNR1 or control

antibody (ctrl) (mGO53) (Wardemann et al. 2003) were added and the spiking activity traced for another 24h (Fig. 1A, I). Remarkably, we observed a dramatic change in network spiking activity in the presence of hNR1 antibody that gradually increased over the period of 24h of antibody treatment, reaching a highly active state (baseline = 1.12 ± 0.47 , after 24h hNR1 = 3.02 ± 0.47 , paired t-test, $p = 0.02$) (Fig. 1B, D). The effect of the control antibody was more limited, eliciting a modest, but not significant, change in network spiking activity during this period (normalized baseline = 1.01 ± 0.01 , after 24h ctrl = 1.72 ± 0.27 , 5, paired t-test, $p = 0.07$) (Fig. 1B, C).

The observed increase in spiking caused by the hNR1 antibody is both surprising and counterintuitive, as earlier studies demonstrated that active neuronal networks commonly respond to blockage of NMDARs - by NMDAR antagonists like AP5 or ketamine - with a rapid decrease of their spiking activity (Teppola et al. 2018; Teppola, Aćimović, and Linne 2019; Emnett et al. 2013), which slowly recovers over hours, due to various slow adaptive processes (Kaufman, Reinartz, and Ziv 2014). Conceptually, this difference could be due to the fact that the hNR1 antibody only blocks 50% of the whole-cell NMDAR currents in cultured hippocampal neurons (Kreye et al. 2016; Hughes et al. 2010), while saturating concentrations of AP5 and ketamine block all active NMDARs. We therefore hypothesized that the hNR1 antibody-induced increase in spiking activity could be an adaptive response of the network to an incomplete block of NMDARs. To test this hypothesis, we carried out similar experiments by adding $1 \mu\text{M}$ of the NMDA antagonist AP5 ($1 \mu\text{M}$ being the IC50 for NMDARs) instead of antibody, to block 50% of the surface NMDA receptors. Here, the presence of $1 \mu\text{M}$ AP5 was associated with a modest, yet non-significant, increase in spiking activity over 24h (baseline = 1.02 ± 0.01 , after 24h $1 \mu\text{M}$ AP5 = 1.43 ± 0.13 , paired t-test, $p = 0.09$) (Fig. 1B, E, yellow data points). As the magnitude of this change was just a fraction of that elicited by hNR1 antibody (~43% vs ~300%, respectively) (Fig. 1D, F), it is possible that the underlying mechanism of the latter could be different, e.g. by affecting a subpopulation of receptors or cell types. Of note, AP5 is expected to act more quickly than the hNR1 antibody, yet as both conditions involved chronic multi-

hour treatments, the observed outcomes are anticipated to be due to more slow adapting processes by both manipulations.

An interesting consequential question is whether networks treated with hNR1 antibody or AP5 are still responsive to pharmacological manipulations of NMDARs. This was examined by adding saturating concentrations of AP5 (50 μ M) at the end of the 24h treatment with hNR1, control antibody or 1 μ M AP5 (Fig. 1A). Interestingly, networks treated with the control antibody or 1 μ M AP5 remained responsive to high concentration of AP5, as their spiking rapidly decreased (95% and 87% reduction, respectively) (Fig. 1B, 1G). Furthermore, spiking only partially recovered to about 60% of their initial value 6h later (ctrl = 61%, AP5 = 45%) (Fig. 1F). In contrast, the spiking activity of cortical cultures treated with hNR1 antibody only partially dropped due to AP5 treatment (42% reduction, Fig. 1G) and quickly recovered to 93% of the activity levels preceding AP5 treatment (Fig. 1H). These remained nearly fourfold higher than those observed in networks exposed to the control antibody or 1 μ M AP5. Together these data indicate that the addition of hNR1 antibody to cortical cultures renders the network largely insensitive to NMDAR blockage with AP5.

Subsequently, we asked whether other parameters crucial for behavior of neuronal network, such as bursting, number of spikes per burst and network synchrony, are also affected by hNR1 antibody treatment. While synchrony and number of spikes per burst were not different between the groups (data not shown), we observed a dramatic increase in network bursting activity due to hNR1 antibody treatment (baseline = 1.04 ± 0.03 ; after 24h hNR1 = 3.22 ± 0.63 , paired t-test, $p = 0.027$) (Fig. 1J, L). Burst rate of both control antibody (baseline = 1 ± 0.03 ; after 24h ctrl = 1.05 ± 0.12 , paired t-test, $p = 0.72$) and 1 μ M AP5 (baseline = 1 ± 0.04 ; after 24h 1 μ M AP5 = 1.36 ± 0.2 , paired t-test, $p = 0.2$) treated networks was not changed (Fig. 1J-N). Intriguingly, the hNR1 antibody-induced increase in burst rate seemed to have a more rapid onset than the increase in neuronal spiking (Fig. 1B, J), reaching a plateau after ~7h of hNR1 antibody treatment (Fig. 1J). This phenomenon of elevated burst rates is a characteristic of networks in which inhibition has been removed (Chen et al.

2012). Interestingly, high concentrations of AP5 had a smaller effect on bursting activity than on spiking. Moreover, its immediate effects (Fig. 1O), or recovery of burst rate 6h later (Fig. 1P), were not different between the treatments.

To summarize, these experiments reveal two unexpected effects of hNR1 antibody on cortical neurons: 1) substantial elevations in cortical network spiking and bursting activity and 2) a partial loss of responsiveness to NMDAR blockage by AP5, a situation that could conceivably both adversely affect network function and drive epileptic brain activity in patients.

hNR1 antibody impairs NMDA-mediated Npas4 expression in cortical cultures

Neuronal networks are complex systems whose function depends on the proper function of various neuronal cell types and regulatory mechanisms, allowing them to maintain their activity within a narrow range. Normally under increasing activity conditions, calcium influx through NMDARs signals to the cell nucleus and triggers an adaptive change in neuronal gene expression profiles, resulting in a homeostatic restoration of activity levels (Flavell and Greenberg 2008; Hardingham and Bading 2003). One such activity-dependent transcription factor, whose expression is partially triggered by calcium influx through NMDARs, is Neuronal PAS domain protein 4 (Npas4) (Bloodgood et al. 2013; Spiegel et al. 2014). It was thus of interest to know whether this transcriptional program still operates following the addition of hNR1 antibody. Specifically, we asked whether the hNR1 antibody-induced increase in network activity (Fig. 1) was associated with a corresponding increase in Npas4 expression (Fig. 2). Surprisingly, treating cortical cultures with hNR1 antibody for 2h, 4h or 24h did not induce the expression of Npas4, as measured by changes in fluorescence intensity of somatic Npas4 in cortical cultured neurons (Fig. 2A-D). These data indicate that a Npas4 mediated homeostatic mechanism is not active and/or cannot be engaged in the presence of the hNR1 antibody. As more direct test of the latter, we asked whether hNR1 antibody disrupts NMDA-

432 mediated regulation of Npas4 expression. As expected under control conditions, the addition of
 433 NMDA to cortical cultures induced significant Npas4 expression ($UT = 1 \pm 0.03$, $NMDA = 2.21 \pm 0.17$, $p <$
 434 0.0001) (Fig. 2E, first and second panel, Fig 2F). However, the pre-treatment with hNR1 antibody for
 435 6h or 24h blocked the increase in somatic Npas4 expression, similarly to networks pretreated with
 436 AP5 ($6h\ NR1 + NMDA = 1.28 \pm 0.04$, $24h\ hNR1 + NMDA = 1.13 \pm 0.03$, $3h\ AP5 + NMDA = 1.47 \pm 0.07$, $p <$
 437 0.0001) (Fig. 2E-F). These data suggest that hNR1 antibody interferes with the capacity of cortical
 438 networks to decrease their activity through NMDA/Npas4 regulated gene expression, an observation
 439 that could help explain why the hNR1 antibody-induced hyperactivity is not down-regulated to
 440 homeostatic levels over time. However, less clear is whether the dysregulation of NMDA/Npas4
 441 regulated gene expression can explain why hNR1 antibody treatment increases network activity in
 442 the first place, or whether other mechanisms are at play that more directly regulate
 443 excitatory/inhibitory balance in these networks.

445 **hNR1 antibody disinhibits cortical networks by specifically impairing output of inhibitory neurons**

446 Proper function of cortical networks is determined by a fine balance between excitation and
 447 inhibition. Previous studies have shown that hypofunction of NMDARs can reduce inhibition and
 448 disinhibit cortical networks (Y. Zhang, Behrens, and Lisman 2008b; Homayoun and Moghaddam
 449 2007a). It thus seemed feasible that the hNR1 antibody-induced hyperactivity is a result of network
 450 disinhibition. To test this hypothesis, we designed a set of calcium imaging and electrophysiological
 451 experiments. For calcium imaging, neurons were initially infected at 4 DIV with a lentivirus
 452 expressing RCaMP (Dana et al. 2016), a genetically encoded calcium indicator, and allowed to grow
 453 for 10 more days (14DIV) before antibody treatment. Cortical networks of neurons were then
 454 subjected to live imaging 24h after treatment with hNR1 or control antibodies. Subsequently,
 455 activity levels were measured by detecting somatic calcium transients of individual cells and
 456 compared to untreated cultures. Similar to MEA recordings (Fig. 1), in cortical cultures treated with

the hNR1 antibody the frequency of somatic calcium events dramatically increased, as compared to both untreated networks and those treated with control antibody (UT = 10.89 ± 0.31 , ctrl = 12.55 ± 0.43 , hNR1 = 14.99 ± 0.37 , $p < 0.0001$) (Fig. 3A-C upper panels, D). Interestingly, in this experimental setup the control antibody (ctrl) also increased the frequency of calcium events compared to untreated cultures ($p = 0.007$), though to a lesser extent than the hNR1 antibody (ctrl: 15%, hNR1: 38% increase) (Fig. 3D). These latter data suggest that this antibody is not without its effects, though the mechanism and its antigen remains unknown.

Given the high spiking and bursting frequency of cortical cultures treated with hNR1 antibody, it was of interest to explore whether such cultures could be further disinhibited, perhaps by disengaging the GABAergic system. This was accomplished by adding bicuculline (30 μ M), a GABA_AR antagonist, at the end of 24h treatment with antibodies and re-imaging activity of the same neurons for a within-cell comparison (Fig. 3A-F). As expected, in untreated and control antibody treated conditions, bicuculline increased the frequency of calcium events (paired t-test, $p < 0.0001$, $p = 0.035$, respectively) (Fig. 3E, 3F). Strikingly, bicuculline failed to further increase the frequency of calcium events in cultures treated with the hNR1 antibody (paired t-test, $p = 0.53$, Fig. 3G). These data imply that critical features of the GABAergic system and/or Ca²⁺-activated potassium channels, also known to be inhibited by bicuculline (Khawaled et al. 1999; Johnston 2013), could contribute to antibody-induced hyper-excitability.

As the GABAergic system is a known key regulator of excitatory and inhibitory balance, we designed several experiments to further explore possible inhibitory dysfunction in the context of hNR1 antibody-disrupted NMDAR signaling. Interestingly, studies on the 'glutamatergic hypothesis of schizophrenia' suggest that hypofunction of inhibitory drive onto excitatory pyramidal cells leads to disinhibition of cortical networks (Homayoun and Moghaddam 2007b), producing psychotic symptoms similar to those observed in patients with NMDAR encephalitis. We therefore asked whether the inhibitory drive onto excitatory neurons is also altered in cortical networks due to hNR1

antibody. Here, we performed a set of patch clamp electrophysiology experiments to record miniature inhibitory post-synaptic currents (mIPSCs) from excitatory neurons in cortical cultures (Fig 4A). Excitatory neurons were identified by infecting WT neurons with pLenti_CamKII_mKate2, a lentivirus expressing mKate2 under the glutamatergic specific CamKII promoter, at DIV 2-4 (Fig. 4B). At 15-18 DIV, we recorded mIPSCs from these neurons, after 24h treatment with either hNR1 or control antibody. We observed that hNR1 antibody treatment significantly decreased mIPSCs frequency (ctrl = 6.42 ± 0.37 , hNR1 = 4.94 ± 0.4 , unpaired t-test, $p = 0.009$) and had a tendency to decrease mIPSCs amplitude (ctrl = 46.89 ± 2.89 , hNR1 = 40.01 ± 2.25 , unpaired t-test, $p = 0.06$) compared to control antibodies (Fig. 4C, D) without affecting charge (Fig. 4F). These data indicate that 24h hNR1 antibody treatment indeed decreased inhibitory drive onto excitatory neurons. To test whether this is caused by an overall reduction of surface GABAR function on post-synaptic excitatory neurons, we measured currents induced by bath application of GABA (5 μ M) using a fast-flow system. However, these GABA-currents were similar between control and hNR1 antibody treated cultures (Fig. 4G, H), indicating that the total pools of GABA_AR on excitatory neurons are not altered.

As network activity is sensitive to both excitation and inhibition, we next explored whether the excitatory drive in neuronal cultures was also altered by hNR1 antibody. This was accomplished by recording miniature excitatory post-synaptic currents (mEPSCs) in the presence of antibodies from both excitatory and inhibitory cortical neurons. Interestingly, the frequency, amplitude and charge of mEPSCs onto either cell type were not affected by hNR1 antibody treatment (Fig. 4I-P). These data indicate that hNR1 antibody specifically reduces the inhibitory drive onto excitatory neurons in a cortical network, which could be causal for its overall disinhibition.

506 hNR1 antibody specifically impairs the function of inhibitory and not excitatory autapses

507 As we observed a decrease in overall inhibition and specific changes in inhibitory neuron output, we
 508 next examined the selective effects of the hNR1 antibody on inhibitory cortical neurons in more
 509 detail. Hence, we performed patch-clamp electrophysiological recordings in an autaptic system,
 510 where single neurons are grown on an astrocytic micro-island, allowing the study of cell-
 511 autonomous effects on either inhibitory or excitatory neurons (Fig. 5A). To easily distinguish
 512 between these two neuronal populations, we employed GAD67-GFP mice, in which inhibitory
 513 neurons expressing GAD67 are fluorescently labeled (Tamamaki et al. 2003). Previous groups
 514 reported no changes in electrophysiological properties of these neurons when compared to WT
 515 neurons (Chang et al. 2014). In initial experiments, we examined whether the addition of hNR1 or
 516 control antibodies (24h) affect the total surface expression of the 3 main groups of ionotropic
 517 receptors responsible for synaptic transmission, by acutely bath-applying NMDA (10 μ M), GABA (5
 518 μ M) or kainate (20 μ M) in 1 second pulses and comparing responses of inhibitory and excitatory
 519 cells. Intriguingly, we observed that in autaptic cultures of cortical neurons, hNR1 antibody
 520 treatment did not affect whole-cell NMDA currents on excitatory neurons (ctrl = 1 ± 0.08 , hNR1 =
 521 1.01 ± 0.11 , unpaired t-test, $p = 0.92$) (Fig 5B), yet it selectively decreased whole-cell NMDA currents
 522 on inhibitory neurons (ctrl = 1 ± 0.1 , hNR1 = 0.71 ± 0.07 , unpaired t-test, $p = 0.018$) (Fig. 5E). At the
 523 same time, total GABA- and kainate-specific currents in either cell type remained unchanged (Fig.
 524 5G, data not shown). Next, we measured evoked synaptic responses by inducing an action potential
 525 in these cells, by the brief somatic depolarization of the neuron, and recording of the post-synaptic
 526 current that followed a few milliseconds afterwards. Intriguingly, on inhibitory neurons, synaptic
 527 inhibitory transmission (IPSCs) was significantly reduced by ~41% (ctrl = 1 ± 0.12 , hNR1 = 0.59 ± 0.11 ,
 528 $p = 0.01$) (Fig. 5F), whereas synaptic excitatory transmission (EPSCs) (ctrl = 1 ± 0.13 , hNR1 = $1.31 \pm$
 529 0.19 , unpaired t-test, $p = 0.19$) (Fig. 5C) and pharmacologically isolated synaptic NMDA currents (ctrl
 530 = 1 ± 0.12 , hNR1 = 1.38 ± 0.23 , unpaired t-test, $p = 0.13$) (Fig. 5D) on excitatory neurons remained
 531 unchanged (Fig. 5C, D). What is more, on inhibitory neurons, the reduction of NMDA-currents and

the amplitude of IPSCs seemed to go hand in hand, as there was a significant correlation between these two parameters among inhibitory cells (linear regression, $R^2 = 0.132$, $p = 0.029$) (Fig. 5H). These data suggest that in autaptic cortical neurons hNR1 antibody treatment primarily affects the function of NMDARs on inhibitory neurons, which in turn reduces inhibitory synaptic output by these cells. To explore the mechanism further, we examined the impact of hNR1 antibody on the spontaneous release of GABA from inhibitory neurons by measuring mIPSCs in these autaptic cultures. Here, we observed a significant decrease in mIPSCs amplitude (Fig. 5K) (ctrl = 43.87 ± 2.81 , hNR1 = 30.72 ± 2.46 , unpaired t-test, $p = 0.0008$) and charge (Fig. 5L) (ctrl = 789.7 ± 76.58 , hNR1 = 551.9 ± 58.94 , unpaired t-test, $p = 0.016$). Similar to neuronal network experiments (Fig. 4G, H), we observed no differences in responses after bath application of GABA between autaptic neurons treated with control or hNR1 antibodies (Fig. 5G). Together, these data indicate that the hNR1 antibody specifically impairs NMDAR- and synaptic transmission of cortical inhibitory, but not excitatory neurons, in a cell-autonomous manner.

Importantly, as we observed a modest effect of the control antibody on neuronal network activity (Fig. 3), we evaluated whether it also exerts any significant effects in autaptic cultures. Here, no differences between untreated and control antibody treated cultures were observed in any of reported measures (whole-cell NMDA, GABA and kainate currents, synaptic NMDA currents and EPSCs/IPSCs) in neither excitatory nor inhibitory neurons (data not shown). These data suggest that the control antibody generally has no direct effect on synaptic function in the autaptic system.

551

552 **hNR1 antibody exerts different effects in cortical vs hippocampal neurons**

The lack of effect of hNR1 antibody on NMDA currents in cortical excitatory neurons was surprising, as previous studies have robustly shown that patients' CSF/IgG reduce NMDA currents of hippocampal pyramidal cells (Kreye et al. 2016; Moscato et al. 2014). To verify that the lack of effect

on cortical neurons is not due to technical limitations of our autaptic assays, we repeated the autaptic recordings on hippocampal excitatory neurons. As reported previously (Kreye et al. 2016), treatment with hNR1 antibody selectively reduced whole-cell NMDA currents (36% decrease, ctrl = 1 ± 0.08 , hNR1 = 0.64 ± 0.06 , unpaired t-test, $p = 0.0008$) (Fig. 6A) and synaptic NMDA currents (43% decrease, ctrl = 1 ± 0.15 , hNR1 = 0.58 ± 0.12 , unpaired t-test, $p = 0.039$) (Fig. 6E) in hippocampal excitatory neurons, without affecting EPSC (Fig. 6D), GABA (Fig. 6B) and kainate (Fig. 6C) currents. Importantly, these results demonstrate that hNR1 antibody shows tissue-specificity, and in cortical cultures selectively affect inhibitory, and not excitatory, neurons.

564

hNR1 antibody binds NMDARs within inhibitory synapses and reduce levels of inhibitory pre-synaptic proteins

The results from both neuronal networks (Fig. 4) and autaptic (Fig. 5) recordings suggest that hNR1 antibody causes a dysfunction of synaptic output of inhibitory neurons, represented by a reduced amplitude and/or frequency of IPSCs and mIPSCs. To explore possible presynaptic effects of the hNR1 antibody, we measured levels of proteins crucial for proper function of inhibitory pre-synapses before and after hNR1 antibody treatment by immunocytochemistry in cortical networks. Additionally, by employing GAD67-GFP mice, we distinguished between inhibitory synapses onto inhibitory (GFP-positive), and excitatory (GFP-negative) neurons (Fig. 7B, C). Two proteins are indispensable for proper neurotransmitter release within GABAergic pre-synapses: glutamate decarboxylase 65 (GAD65), which synthesizes GABA from glutamate within pre-synaptic boutons, and the vesicular GABA transporter (VGAT/VIAAT), which subsequently loads GABA into synaptic vesicles. Using quantitative immunocytochemistry, we measured the intensities of fluorescently labelled VGAT and GAD65 puncta along dendrites, to quantify their expression levels. Remarkably, a 24h treatment with hNR1 antibody significantly reduced intensities of VGAT puncta (UT = 1 ± 0.05 , 24h hNR1 = 0.82 ± 0.04 , unpaired t-test, $p = 0.005$) (Fig. 7D, E). Moreover, this decrease was specific

581 to inhibitory synapses onto excitatory neurons, and not onto inhibitory neurons (Fig. 7D, F). On the
 582 other hand, the intensities of GAD65 puncta were unchanged after 24h of control antibody
 583 treatment (Fig. 7G-I). We then additionally measured GAD65 expression levels after 6h of hNR1
 584 antibody treatment. At this time-point, we observed a significant decrease in GAD65 puncta
 585 intensity (UT = 1 ± 0.03 , 6h hNR1 = 0.89 ± 0.02 , 24h hNR1 = 0.95 ± 0.02 , ANOVA Tukey's multiple-
 586 comparison test, $p = 0.01$) (Fig. 7H), which again was specific to inhibitory synapses onto excitatory,
 587 but not inhibitory, neurons (Fig. 7G-I). Together, these data demonstrate that the hNR1 antibody
 588 reduces levels of the pre-synaptic proteins VGAT and GAD65, specifically in inhibitory-to-excitatory
 589 neuron synapses.

590 Finally, we asked if the observed specific effect of hNR1 antibody on inhibitory neurons can be
 591 explained by a distinct binding pattern of the antibody in cortical cultures. One possibility could be
 592 that the antibody binds specifically to inhibitory neurons. Alternatively, the hNR1 antibody could
 593 target different sub-cellular pools of NMDARs, or even bind directly to inhibitory synapses to affect
 594 their function locally. We explored these options by analyzing the acute binding patterns of hNR1
 595 antibody in our cortical cultures. Firstly, live neurons were incubated with hNR1 antibody for 20
 596 minutes at 10°C, washed, and incubated with anti-human secondary antibody for 20 minutes at
 597 10°C. Cells were then fixed and co-stained for the excitatory presynaptic marker VGLUT1 to verify if
 598 hNR1 antibody binds to excitatory synapses, as expected. The degree of co-localization of the hNR1
 599 antibody puncta with VGLUT1 signal was used as a measure of excitatory synaptic vs extra-synaptic
 600 antibody binding. Although the hNR1 antibody decorated all neurons present in the culture in a
 601 punctate pattern, there was a striking difference between the binding patterns on excitatory and
 602 inhibitory neurons. While on inhibitory neurons most of hNR1 antibody puncta (70%) co-localized
 603 with VGLUT1, on excitatory neurons only half of the hNR1 antibody puncta co-localized with VGLUT1
 604 (excit. = $47.28\% \pm 4.53$, inhibit. = $69.75\% \pm 4.19$, unpaired t-test, $p = 0.006$) (Fig. 8A, B), suggesting
 605 that on excitatory neurons a larger pool of hNR1 antibody binds to structures outside of VGLUT1-
 606 positive synapses. Next, we asked if hNR1 antibody can also bind NMDARs associated with inhibitory

synapses. Here, neurons were co-stained with the hNR1 antibody and the inhibitory post-synaptic marker gephyrin to assess the degree of co-localization. Using confocal microscopy and z-stack analysis, we observed that hNR1 antibody indeed binds to a sub-population of inhibitory synapses in our cortical cultures (Fig. 8C, D). Remarkably, up to 30% of inhibitory synapses on excitatory neurons bound the hNR1 antibody, whereas this phenomenon was much less common for inhibitory synapses on inhibitory neurons (excit. = $30.84\% \pm 2.05$, inhibit. = $11.17\% \pm 1.52$, unpaired t-test, $p < 0.0001$) (Fig. 9E). This preference raises the possibility that the observed functional impact of the hNR1 antibody on inhibitory synapses may be due to the direct effects of this antibody on these synapses.

6. DISCUSSION

Our study demonstrates that a patient-derived antibody targeting the NR1 subunit of NMDARs adversely affects the function of cortical neuronal networks, driving them into a hyper-active state (Fig. 1, 3). This increased excitability seems to be a result of reduced inhibitory neuron output onto excitatory neurons, as indicated by reduced amplitudes of IPSCs, lowered frequency of network mIPSCs and decreased levels of inhibitory pre-synaptic proteins (Fig. 9). This contrasts with the effects of this antibody on hippocampal neurons, where it primarily affects NMDAR function on excitatory neurons (Fig 6, Kreye et al., 2016). Moreover, in cortical networks hNR1 antibody interferes with NMDAR-Npas4-mediated mechanisms of stabilizing network excitability (Fig. 2). These observations provide insights into how such autoantibodies can differentially affect hippocampal versus cortical circuits, giving rise to impaired memory function or psychiatric symptoms in patients with NMDAR encephalitis, respectively.

631 **hNR1 antibody increases cortical network activity**

632 Our primary observation is that hNR1 antibody causes a hyper-excitabile state of cortical networks
 633 (Fig. 1, 3). Although counterintuitive, since NMDAR hypofunction should dampen glutamatergic
 634 transmission, our data are in line with clinical and molecular studies showing that seizures occur in
 635 ~60% of patients with NMDAR encephalitis (Bruijn et al. 2019) and that seizure susceptibility is
 636 increased in mice exposed to patient CSF (Wright et al. 2015).

637 Intriguingly, in contrast to our findings (Fig. 1), two groups used MEAs to assess effects of patient CSF
 638 on cortical (Jantzen et al. 2013) or hippocampal (Koch et al. 2019) neuronal networks and reported a
 639 relative decrease in activity. Such differences could arise from varied approaches used. Both studies
 640 examined the acute effects of antibodies (10-15 mins), using patient CSF containing a mixture of
 641 antibodies. In contrast, our 46h recording revealed that the presence of the hNR1 antibody leads to a
 642 gradually increasing activity, however over 24h and not 15 min. Mechanistically, this occurs in a
 643 timeframe corresponding to the rate of antibody-induced receptor internalization both described
 644 previously (Moscato et al. 2014) and observed for hNR1 (data not shown). Thus, the increase in
 645 cortical network activity by the hNR1 antibody seems to be a relevant causal mechanism for both
 646 hyper-excitability and epilepsy in patients.

647 A fundamental question raised by our MEA analysis is how the hNR1 antibody leads to a steady
 648 increase in network spiking? One possibility is an adaptive response of the network to a partial block
 649 of NMDARs. Blocking half of surface NMDARs (1 μ M AP5) lead to a modest but non-significant
 650 increase in network spiking, indicating that it is possible, yet unlikely, that a partial block of surface
 651 NMDARs contributes to the seen increase in network activity. Rather, these data suggest that hNR1
 652 antibody might instead act through specific receptors/neuronal subtypes to disturb E/I balance.
 653 Indeed, in the presence of hNR1 antibody, cortical networks dramatically increase their bursting
 654 activity (Fig. 1J-P) and loose responsiveness to bicuculline (Fig. 3A-F), suggesting that the inhibitory
 655 system is likely affected.

656 A second puzzling question is why neuronal spiking continues to climb in the presence of hNR1
657 antibody, when one would expect mechanisms stabilizing network excitability, such as Npas4
658 signaling (Bloodgood et al. 2013), to counter a hyperactive state. Surprisingly, nuclear Npas4 levels
659 did not increase in the presence of hNR1 antibody (Fig. 2A-D), suggesting that this mechanism
660 became ineffective. Accordingly, normal NMDA-mediated increase in nuclear Npas4 was blocked by
661 addition of hNR1 antibody (Fig. 2E-F). This could be due to a direct effect of hNR1 antibody on
662 NMDARs, as AP5 also prevented the induction of Npas4 after NMDA application (Fig. 2E-F). Together,
663 these data indicate that the hNR1 antibody can interfere with homeostatic mechanisms regulated by
664 NMDAR/Npas4 signaling.

665

666 **hNR1 antibody impairs the output of cortical inhibitory neurons**

667 While the depression of NMDAR/Npas4 signaling may explain why hyper-excitability is not scaled
668 down, it does not fully explain why the activity increases in the first place. One possibility is that
669 hNR1 antibody acts on a subset of receptors, synapses or neurons. In the cortex, a correct E/I
670 balance is maintained by GABAergic interneurons orchestrating excitability of pyramidal cells. Our
671 data indicate that cortical GABAergic interneurons are indeed a primary target of hNR1 antibody,
672 specifically altering the output of inhibitory synapses onto excitatory neurons. Consistently, hNR1
673 antibody treatment decreased action potential-independent, tonic inhibitory drive onto excitatory
674 neurons, measured by frequency of mIPSCs (Fig. 4A-F). This was specific to inhibitory synapses, as
675 mEPSCs frequency remained unchanged (Fig. 4I-P). This is further supported by a decrease in the
676 levels of both VGAT and GAD65 in presynaptic inhibitory boutons formed onto excitatory neurons,
677 but not inhibitory interneurons (Fig. 7).

678 Importantly, cell-autonomous autaptic recordings confirmed the specific impact of hNR1 antibody on
679 inhibitory neuron function, where it significantly reduced NMDA- and evoked synaptic currents

680 exclusively in inhibitory autaptic neurons (Fig. 5). Although reduced NMDA-currents are likely due to
 681 receptor internalization, how loss of surface NMDARs translates into reduced inhibitory synaptic
 682 transmission remains elusive. Conceivably, reduced NMDAR-mediated Ca^{2+} influx could trigger
 683 changes in transcriptional profiles in these neurons, leading to decreased levels of pre-synaptic
 684 proteins (Fig. 7) and ultimately synaptic output (Fig. 5), similarly to networks in which excitation has
 685 been removed (Lau and Murthy 2012). However, given that we observed increased somatic calcium
 686 signals in all cortical neurons (Fig. 3), whether such mechanisms are involved remains unclear and
 687 further RNA sequencing studies are needed to elucidate possible cell-type specific transcriptional
 688 changes.

689 **hNR1 antibody shows brain regional specificity**

690 Remarkably, our data implies that autoantibodies can have different effects in varying brain regions.
 691 In cortex, we find that hNR1 specifically impairs cortical inhibitory, but not excitatory, neurons. This
 692 contrasts with studies in the hippocampus, showing that these autoantibodies primarily affect
 693 excitatory neuron function (Fig. 6, (Kreye et al. 2016; Hughes et al. 2010). This regional specificity
 694 indicates that there are limitations with current pre-clinical models, which passively deliver patient
 695 CSF/antibodies into ventricles (Planagumà et al. 2016; Taraschenko et al. 2019) or hippocampus
 696 (Kersten et al. 2019; Würdemann et al. 2016). While this approach can provide direct antibody access
 697 to hippocampal structures, they rarely reach cortical regions (Planagumà et al. 2015). As such, active
 698 immunization models which more often report increased sensitivity to seizures (Jones et al. 2019;
 699 Wagnon et al. 2020) may provide for a more clinically-relevant distribution of antibodies and thus a
 700 better understanding of their region-specific effects.

701 **hNR1 antibody binds specific pools of NMDARs and affects inhibitory synapses**

702 At present, it is unclear which mechanisms lead to brain regional and/or neuronal type specificity of
 703 hNR1 antibody. One possibility could be that the hNR1 antibody binds variably on excitatory versus

704 inhibitory neurons. Acute antibody binding studies supports this idea, as hNR1 antibody differentially
 705 binds synaptic and extra-synaptic NMDARs on inhibitory vs excitatory cortical neurons (Fig. 8A-B).
 706 This is relevant as synaptic and extra-synaptic NMDARs trigger distinct downstream signaling
 707 pathways and could therefore contribute to differential effects on each cell type.

708 An intriguing complementary mechanism could involve local effects of hNR1 antibody on pre-
 709 synaptic NMDARs. Although controversial and debated in the field, pre-synaptic NMDARs within
 710 inhibitory synapses have been shown to control neurotransmitter release (Bouvier et al. 2015) and
 711 regulate inhibitory drive onto pyramidal neuron in prefrontal cortex (Pafundo et al. 2018; Mathew
 712 and Hablitz 2011). Thus, their hypofunction due to hNR1 binding could partially explain the observed
 713 decrease in mIPSCs (Fig. 4). Accordingly, we observed that the hNR1 antibody indeed decorates
 714 inhibitory synapses (Fig. 8). While it is unclear whether this binding is pre- or post-synaptic, a recent
 715 immuno-EM study found that the hNR1 antibody could indeed decorate presynaptic-NMDARs
 716 (Wagner et al. 2020). Importantly, studies by Fiszman and colleagues reported that reducing
 717 presynaptic-NMDAR signaling decreases the size of pre-synaptic inhibitory boutons, as measured by
 718 GAD65 puncta intensity, similar to our observations (Fig. 7). Thus, in addition to more general effects
 719 of the hNR1 antibodies on inhibitory interneurons, there is an emerging framework for how these
 720 antibodies could also have more selective actions on subsets of inhibitory synapses, which ultimately
 721 alter the excitability of cortical networks in patients with NMDAR encephalitis.

722

723 **Relevance for neuropsychiatric disorders**

724 Remarkably, NMDAR hypofunction on cortical interneurons has long been implicated to underlie
 725 cortical disinhibition and core psychiatric symptoms in schizophrenia (Nakazawa et al. 2012). Genetic
 726 deletion of NMDARs in interneurons *in vivo* produces schizophrenia-like behaviors (Belforte et al.
 727 2010), impairs E/I balance and increases excitability of cortical pyramidal cells (Pafundo et al. 2021).

728 Similarly, ketamine impairs mIPSCs, without affecting mEPSCs, and leads to increased pyramidal
729 excitability in prefrontal cortical slices (Y. Zhang, Behrens, and Lisman 2008), which is strikingly
730 similar to our effects after hNR1 antibody treatment (Fig. 1, 3, 4). Our data thus provides evidence
731 that patient-derived autoantibodies can disturb similar pathways and suggest a possible common
732 mechanism across neuropsychiatric disorders.

733 Importantly, the proposed mechanisms of antibody action are not mutually exclusive and may act in
734 a complementary manner to exert regional changes in synaptic plasticity, subunit-specific
735 transcriptional changes and presynaptic regulation of inhibitory drive to produce complex behavioral
736 and cognitive phenotypes. Together, our data reveals a novel, region-specific mechanism of anti-
737 NMDAR antibodies in cortical neurons causing inhibitory dysfunction and network disinhibition.

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971 **Figure 1. hNR1 antibody increases spiking and bursting activity of cortical cultures. (A, I)** Experimental design
 972 of multi-electrode array (MEA) experiments. Baseline activity of cortical networks was recorded for 15h,
 973 followed by addition of 1ug/ml of hNR1 antibody, control antibody or 1μM of AP5 and 24h recording of activity
 974 in the presence of antibodies/1μM AP5. After 24h of treatment, saturating concentration of AP5 (50 μM) was
 975 added to the network and another 6h of activity were recorded. **(B)** Normalized spike rate of neuronal
 976 networks. Each data point represents a number of spikes recorded within a 10 min interval from all 59
 977 electrodes and normalized to the last 3h of baseline activity. Addition of hNR1 antibody (green data points),
 978 and to a much lesser degree control (ctrl, mGO53) antibody (grey data points) or AP5 at its IC50 concentration
 979 (1 μM) (yellow data points), increase spiking of the network. hNR1 = 5 independent experiments, ctrl = 5
 980 independent experiments, AP5 = 3 independent experiments. Error bars indicate SEM. **(C-E)** Within-dish
 981 comparison of normalized spike rate of the same network at the end of baseline recording (1h) and after 24h of
 982 treatment (1h) tested by paired t-test. **(C)** baseline = 1.01 ± 0.01 , after 24h ctrl = 1.72 ± 0.27 , 5, $p = 0.07$; **(D)**
 983 baseline = 1.12 ± 0.47 , after 24h hNR1 = 3.02 ± 0.47 , $p = 0.02$; **(E)** baseline = 1.02 ± 0.01 , after 24h 1 μM AP5 =
 984 1.43 ± 0.13 , $p = 0.09$. **(F-H)** Percentage increase in normalized spike rate at the end (10 min) or beginning (10
 985 min) of respective treatment. Each data point represents individual network (MEA dish). Percentage increase in
 986 spike rate: **(F)** at the end of baseline compared to end of antibody treatment, ctrl = $71.71\% \pm 27.46$; hNR1 =
 987 $200.5\% \pm 42.96$, 1 μM AP5 = $42.4\% \pm 10.76$, ctrl vs hNR1 $p = 0.045$, hNR1 vs 1 μM AP5 $p = 0.034$; **(G)** at the end
 988 of antibody treatment compared to beginning of 50 μM AP5, ctrl = $-94.96\% \pm 5.03$; hNR1 = $-41.69\% \pm 15.88$; 1
 989 μM AP5 = $-86.51\% \pm 1.33$, ctrl vs hNR1 $p = 0.085$; **(H)** at the end of antibody treatment compared to recovery
 990 6h after addition of 50 μM AP5, ctrl = $61.34\% \pm 10.65$; hNR1 = $92.65\% \pm 8.8$; 1 μM AP5 = $45.04\% \pm 4.88$, hNR1
 991 vs 1 μM AP5 $p = 0.038$. **(J)** Normalized burst rate of neuronal networks. Each data point represents a number of
 992 bursts recorded within a 10 min interval, normalized to the last 3h of baseline. Network bursting increases in
 993 the presence of hNR1 antibody, but not of control antibody nor 1 μM AP5. **(K-M)** Within-dish comparison of
 994 normalized burst rates of the same networks at the end of baseline recording (1h) and after 24h of treatment
 995 (1h) tested by paired t-test. **(K)** baseline = 1 ± 0.03 ; after 24h ctrl = 1.05 ± 0.12 , $p = 0.72$; **(L)** baseline = $1.04 \pm$
 996 0.03 ; after 24h hNR1 = 3.22 ± 0.63 , $p = 0.027$; **(M)** baseline = 1 ± 0.04 ; after 24h 1 μM AP5 = 1.36 ± 0.2 , $p = 0.2$.
 997 **(N-P)** Percentage increase in normalized burst rate at the end (10 min) or beginning (10 min) of respective
 998 treatments. Percentage increase in burst rate: **(N)** at the end of baseline compared to end of antibody

999 treatment, ctrl = $23.31\% \pm 19.47$; hNR1 = $247.5\% \pm 66.75$; 1 μM AP5 = $30.14\% \pm 25.57$, ctrl vs hNR1 $p = 0.01$,
1000 hNR1 vs 1 μM AP5 $p = 0.03$; **(O)** end of antibody treatment compared to beginning of 50 μM AP5, ctrl = -
1001 $70.46\% \pm 9.9$; hNR1 = $-36.58\% \pm 25.24$; 1 μM AP5 = $-26.05\% \pm 25.24$; **(P)** end of antibody treatment compared
1002 to recovery 6h after addition of 50 μM AP5, ctrl = $147\% \pm 64.39$; hNR1 = $149.6\% \pm 16.73$; 1 μM AP5 = $143\% \pm$
1003 26.56 . Error bars indicate SEM. Paired t-test (C-E, K-M) or ANOVA Tukey's multiple-comparison test (F-H, N-P)
1004 were used to evaluate statistical significance. * $p < 0.05$.

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1020 **Figure 2. hNR1 antibody dysregulates NMDA-induced expression of Npas4, a transcription factor regulating**
 1021 **network activity. (A-D)** Cortical neuronal cultures 14-16 DIV were incubated with 1 $\mu\text{g}/\text{ml}$ of hNR1 antibody for
 1022 2h, 4h or 24h, then fixed and stained with anti-Npas4 antibody to measure intensity of somatic Npas4
 1023 expression. Neither short-term (2h, 4h, A) nor long-term (24h, C) treatment with hNR1 induced somatic Npas4
 1024 expression. **(B, D)** Normalized intensity of somatic Npas4 signal from 3 independent experiments: (B) UT = $1 \pm$
 1025 0.03 , n = 218 neurons; 2h hNR1 = 0.97 ± 0.04 , n = 133 neurons; 4h hNR1 = 1.08 ± 0.03 , n = 168 neurons. (D) UT
 1026 = 1 ± 0.04 , n = 280 neurons; 24h hNR1 = 0.98 ± 0.03 , n = 183 neurons. **(E)** Stimulation of neurons with 2 μM
 1027 NMDA for 2h induces activity-driven increase of Npas4 expression compared to untreated condition. 6h or 24h
 1028 pretreatment with hNR1 antibody (1 $\mu\text{g}/\text{ml}$), as well as 3h pretreatment with NMDAR antagonist AP5 (50 μM),
 1029 blocks NMDA-induced expression of Npas4. **(F)** Normalized intensity of somatic Npas4 signal from 3
 1030 independent experiments: UT = 1 ± 0.03 , n = 235 neurons; NMDA = 2.21 ± 0.17 , n = 197 neurons; 6h NR1 +
 1031 NMDA = 1.28 ± 0.04 , n = 172 neurons; 24h hNR1 – NMDA = 1.13 ± 0.03 , n = 179 neurons; 3h AP5 + NMDA =
 1032 1.47 ± 0.07 , n = 166 neurons. Error bars indicate SEM. Scale bar = 100 μm . ANOVA Tukey's multiple-comparison
 1033 test (B, F) or paired t-test (D) were used to evaluate statistical significance. ****p<0.0001.

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Figure 3. Calcium imaging reveals that networks treated with hNR1 antibody cannot be further disinhibited by bicuculline. (A-C) Representative raster plots of calcium events recorded from neuronal somas (neuron ID on the y axis) expressing RCamp in untreated condition (A, upper panel), after 24h of control antibody (B, upper panel) or 24h of hNR1 antibody (C, upper panel) treatment. Lower panels in (A-C) show representative raster plots of calcium events of the same neurons as in upper panels following addition of bicuculline (30 μ M) at the end of respective treatment. (D) Frequency of somatic calcium events in untreated condition, after 24h of hNR1 or control antibodies treatment (upper panels in A-C); UT = 10.89 ± 0.31 , n = 366 cells, ctrl = 12.55 ± 0.43 , n = 277 cells, hNR1 = 14.99 ± 0.37 , n = 391 cells, 6 independent experiments, **p = 0.007, ****p < 0.0001. (E-G) Within-cell comparison of the frequency of calcium events in the same cells at the end of 24h treatment and after addition of bicuculline tested by paired t-test from 3 independent experiments reveals that bicuculline disinhibits the networks in untreated condition (D) and after control antibodies treatment (E) but not after hNR1 (F) treatment. Quantification of calcium events: (E) UT = 4.83 ± 0.51 , UT + Bic = 10.87 ± 0.81 , mean of difference = 6.05 ± 0.75 , n = 47 neurons, p < 0.0001; (F) 24h ctrl = 6.84 ± 0.074 , 24h ctrl + Bic = 8.61 ± 0.59 , mean of difference = 1.76 ± 0.82 , n = 80 neurons, p = 0.035; (G) 24h hNR1 = 9.88 ± 0.88 , 24h hNR1 + Bic = 10.24 ± 0.45 , mean of difference = 0.57 ± 0.9 , n = 62 neurons, p = 0.53. Error bars indicate SEM. ANOVA Tukey's multiple-comparison test (D) and paired t-test (E-G) were used to evaluate statistical significance. *p<0.05, **p<0.01, ****p<0.0001, ns = not significant.

1070 **Figure 4. hNR1 antibodies decrease inhibitory drive onto excitatory neurons in cortical circuits, but do not**
1071 **affect excitatory drive. (A-H)** Inhibitory drive onto excitatory neurons was measured by miniature inhibitory
1072 post-synaptic currents (mIPSCs) recorded from excitatory neurons in cortical neuronal networks. **(A)** Schematic
1073 model of a simplified neuronal network composed of excitatory pyramidal neurons (red) and inhibitory
1074 neurons (blue), forming different types of synapses onto each other. Inhibitory synapse onto excitatory neuron
1075 is marked with a circle as a synapse of interest measured in this experiment. **(B)** mIPSCs were recorded from
1076 excitatory neurons identified by expression of mKate2 expressed under excitatory neuron CamKII promoter.
1077 Left panel: BF image of representative cortical neuronal network, right panel: excitatory neuron identified by
1078 mKate2 fluorescent signal. **(C)** Representative traces of mIPSCs recorded in a whole-cell patch-clamp
1079 configuration from excitatory neurons after 24h of treatment with control (upper panel) or hNR1 (lower panel)
1080 antibodies. Treatment with hNR1 antibody decreases frequency of mIPSCs onto excitatory neurons **(D)** and has
1081 tendency to decrease their amplitude **(E)**, without affecting their charge **(F)**. **(D-F)** Quantification of mIPSCs
1082 parameters from 3 independent experiments, ctrl n = 33 neurons, hNR1 n = 34 neurons: **(D)** mIPSCs frequency:
1083 ctrl = 6.42 ± 0.37 , hNR1 = 4.94 ± 0.4 , $p = 0.009$; **(E)** mIPSCs amplitude: ctrl = 46.89 ± 2.89 , hNR1 = 40.01 ± 2.25 ,
1084 $p = 0.06$; **(F)** mIPSCs charge: ctrl = 606.5 ± 29.72 , hNR1 = 610 ± 36.37 . **(G-H)** hNR1 antibody treatment does not
1085 affect levels of GABA receptors on the post-synaptic excitatory neurons from which mIPSCs were recorded. **(G)**
1086 Representative traces of GABA currents recorded from excitatory neurons, evoked by 1s pulse application of 5
1087 μ M GABA. **(H)** Quantification of GABA current amplitude presented in (F): ctrl = 6.84 ± 1.1 , n = 23 neurons,
1088 hNR1 = 7.6 ± 1.04 , n = 7.6 ± 1.04 , n = 25 neurons. **(I-P)** hNR1 antibody does not affect excitatory miniature
1089 transmission (mEPSCs) onto neither inhibitory (I-L) nor excitatory (M-P) neurons. **(I, M)** Representative traces of
1090 mEPSCs recorded from inhibitory (I) or excitatory (M) neurons. Quantification of mEPSCs parameters from 3
1091 independent experiments: **(J)** mEPSCs frequency: ctrl = 9.83 ± 1.41 , n = 27 neurons, hNR1 = 9.17 ± 1.2 , n = 26
1092 neurons; **(K)** mEPSCs amplitude: ctrl = 36.7 ± 2.71 , n = 26 neurons, hNR1 = 34.41 ± 2.68 , n = 26 neurons; **(L)**
1093 mEPSCs charge: 97.83 ± 5.47 , n = 26 neurons, hNR1 = 91.52 ± 4.02 , n = 24 neurons; **(N)** mEPSCs frequency: ctrl
1094 = 1.32 ± 0.23 , n = 28 neurons, hNR1 = 1.98 ± 0.21 , n = 28 neurons; **(O)** mEPSCs amplitude: ctrl = 20.09 ± 0.93 , n
1095 = 25 neurons, hNR1 = 19.84 ± 0.92 , n = 28 neurons; **(P)** mEPSCs charge: ctrl = 66.76 ± 2.96 , n = 23 neurons,
1096 hNR1 = 64.86 ± 3.22 , n = 24 neurons). Error bars indicate SEM. Unpaired t-test was used to evaluate statistical
1097 significance. ** $p < 0.01$.

1098 **Figure 5. hNR1 antibody impairs function of cortical inhibitory, but not excitatory neurons, in a cell-**
 1099 **autonomous manner. (A)** Example image of a single cortical neuron grown on an astrocytic island in autaptic
 1100 culture and a schematic representation of a patch pipette approaching the neuron. **(B-D) Lower panels:**
 1101 representative traces of (B) whole-cell NMDA currents evoked by 1s bath application of NMDA (10 μ M), (C)
 1102 evoked excitatory post-synaptic currents (EPSCs), (D) evoked synaptic NMDA currents in excitatory neurons
 1103 after 24h treatment with control or hNR1 antibodies. *Upper panels:* Normalized current amplitudes from 4
 1104 independent experiments on excitatory neurons **(B):** ctrl = 1 ± 0.08 , n = 32 neurons, hNR1 = 1.01 ± 0.11 , n = 32
 1105 neurons; **(C):** ctrl = 1 ± 0.13 , n = 33 neurons, hNR1 = 1.31 ± 0.19 , n = 31 neurons; and **(D):** ctrl = 1 ± 0.12 , n = 29
 1106 neurons, hNR1 = 1.38 ± 0.23 , n = 22 neurons. **(E-G) Lower panels:** representative traces of (E) whole-cell NMDA
 1107 currents, (F) evoked inhibitory post-synaptic currents (IPSCs), (G) whole-cell GABA currents evoked by 1s bath
 1108 application of GABA (5 μ M) of inhibitory neurons after 24h treatment with control or hNR1 antibodies. *Upper*
 1109 *panels:* normalized current amplitudes from 4 independent experiments: **(E)** ctrl = 1 ± 0.1 , n = 31 neurons,
 1110 hNR1 = 0.71 ± 0.07 , n = 36 neurons, p = 0.018; **(F)** ctrl = 1 ± 0.12 , n = 35 neurons, hNR1 = 0.59 ± 0.11 , n = 37
 1111 neurons, p = 0.014; **(G)** ctrl = 1 ± 0.1 , n = 25 neurons, hNR1 = 0.81 ± 0.11 , n = 26 neurons. **(H)** Correlation
 1112 between normalized IPSC and NMDA current amplitudes of individual inhibitory neurons. Linear regression: R^2
 1113 = 0.132, p = 0.029. **(I-L)** Analysis of mIPSCs recorded from autaptic inhibitory neurons after 24h of control or
 1114 hNR1 antibodies treatment, 4 independent experiments. **(I)** Representative traces of mIPSCs. **(J)** mIPSCs
 1115 frequency: ctrl = 1.66 ± 0.37 , n = 31 neurons, hNR1 = 1.08 ± 0.21 , n = 35 neurons. **(K)** mIPSCs amplitude: ctrl =
 1116 43.87 ± 2.81 , n = 29 neurons, hNR1 = 30.72 ± 2.46 , n = 32 neurons, p = 0.0008. **(L)** mIPSCs charge: ctrl = $789.7 \pm$
 1117 76.58 , n = 29 neurons, hNR1 = 551.9 ± 58.94 , n = 32 neurons, p = 0.016. Error bars indicate SEM. Unpaired t-
 1118 test (B-D, E-G, J-L) and linear regression (H) were used to evaluate statistical significance. *p<0.05, ***p<0.001.

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1124 **Figure 6. hNR1 antibody impairs NMDA currents of excitatory hippocampal neurons.** In hippocampal autaptic
 1125 cultures, 24h treatment with hNR1 antibody (1 μ g/ml) selectively decreases whole-cell (A) and synaptic (E)
 1126 NMDA currents of excitatory neurons. **(A-C)** Whole-cell receptor currents were evoked by 1s bath application of
 1127 **(A)** NMDA (10 μ M), **(B)** GABA (30 μ M) or **(C)** kainate (20 μ M). Quantification of normalized current amplitudes
 1128 from 3 independent experiments: **(A)** NMDA currents: ctrl = 1 ± 0.08 , n = 30 neurons, hNR1 = 0.64 ± 0.06 , n =
 1129 23 neurons, p = 0.0008; **(B)** GABA currents: ctrl = 1 ± 0.08 , n = 31 neurons, hNR1 = 0.94 ± 0.09 , n = 22 neurons;
 1130 **(C)** kainate currents: ctrl = 1 ± 0.14 , n = 21 neurons, hNR1 = 0.83 ± 0.15 , n = 16 neurons. **(D-E)** Synaptic
 1131 responses were evoked by a brief somatic depolarization of neurons from -70 mV to 0 mV for 2 ms, synaptic
 1132 NMDA currents were recorded in the presence of 0 mM Mg²⁺, 10 μ M glycine and 10 μ M NBQX. Quantification
 1133 of normalized current amplitudes from 3 independent experiments: **(D)** EPSCs: ctrl = 1 ± 0.13 , n = 31 neurons,
 1134 hNR1 = 0.99 ± 0.14 , n = 23 neurons; **(E)** synaptic NMDA: ctrl = 1 ± 0.15 , n = 26 neurons, hNR1 = 0.58 ± 0.12 , n =
 1135 20 neurons, p = 0.039. Error bars indicate SEM. Unpaired t-test was used to evaluate statistical significance.
 1136 *p<0.05, ***p<0.001.

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1150 **Figure 7. hNR1 antibodies decrease levels of inhibitory pre-synaptic proteins VGAT and GAD65 in inhibitory-**
 1151 **to-excitatory neuron synapses. (A)** Schematic model showing investigated inhibitory synapses and
 1152 summarizing the finding: decreased levels of GAD65 and VGAT at inhibitory synapses onto excitatory (yellow
 1153 circle), but not inhibitory (green circle), neurons. **(B-C)** Representative images of cortical excitatory (B) and
 1154 inhibitory (C) neurons identified by staining with antibodies against GFP. Only proximal dendrites close to soma,
 1155 which are easily identified as GFP-positive or GFP-negative, were chosen for ROI selection (boxes). **(D, G)**
 1156 Images of ROIs of cortical excitatory and inhibitory neurons which were fixed and stained with antibodies
 1157 against GFP, MAP2 and either VGAT (D) or GAD65 (G), in untreated condition or after 6h or 24h of hNR1
 1158 antibodies treatment. **(E-F)** Normalized intensity of VGAT puncta along dendrites from 3 independent
 1159 experiments: **(E)** on excitatory neurons: UT = 1 ± 0.05 , n = 56 ROIs, 24h hNR1 = 0.82 ± 0.04 , n = 55 ROIs, p =
 1160 0.005. **(F)** on inhibitory neurons: UT = 1 ± 0.04 , n = 47 ROIs, 24h hNR1 = 0.96 ± 0.03 , n = 57 ROIs. **(H-I)**
 1161 Normalized intensity of GAD65 puncta along dendrites from 3 independent experiments: (H) on excitatory
 1162 neurons: UT = 1 ± 0.03 , n = 64 ROIs, 6h hNR1 = 0.89 ± 0.02 , n = 59 ROIs, 24h hNR1 = 0.95 ± 0.02 , n = 67 ROIs, p
 1163 = 0.01; (I) on inhibitory neurons: UT = 1 ± 0.02 , n = 64 ROIs, 6h hNR1 = 0.94 ± 0.03 , n = 53 ROIs, 24h hNR1 =
 1164 0.96 ± 0.03 , n = 58 ROIs. Error bars indicate SEM. Scale bar = 10 μ m. Paired t-test (E-F) and ANOVA Tukey's
 1165 multiple-comparison test (H-I) were used to evaluate statistical significance. *p<0.05, **p<0.01.

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1175 **Fig 8. hNR1 antibody binds inhibitory synapses, preferentially on excitatory neurons.** (A) Images of cortical
 1176 neurons which were live stained with hNR1 antibody to detect acute binding pattern of the antibodies, and
 1177 then fixed and co-stained for excitatory synaptic marker VGLUT1. Degree of colocalization of hNR1 antibody
 1178 puncta and VGLUT1 puncta was used as a measure of excitatory synaptic or extra-synaptic staining. (B)
 1179 Percentage of hNR1 antibody puncta positive for VGLUT1 (synaptic staining) per ROI: excitatory neurons =
 1180 47.28 ± 4.53 , $n = 28$ ROIs, inhibitory neurons = 69.75 ± 4.19 , $n = 35$ ROIs, 2 independent experiments, $p =$
 1181 0.006 . (C) Images of cortical neurons live stained with hNR1 antibodies for acute hNR1 antibodies binding
 1182 pattern, which were then fixed and co-stained with inhibitory synaptic marker gephyrin. Degree of
 1183 colocalization between hNR1 antibody puncta and gephyrin puncta was used to assess inhibitory synaptic
 1184 binding of the antibodies. Arrowheads indicate overlap between hNR1 and gephyrin. (D) Lower panel: 3D (z
 1185 stack) volume images of individual inhibitory synapses from images in the upper panel at the level of yellow
 1186 line, representing cross-section through 12 planes of z-stack. (E) Percentage of gephyrin puncta positive for
 1187 hNR1 antibodies from 3 independent experiments: excitatory neurons = 30.84 ± 2.05 , $n = 53$ ROIs, inhibitory
 1188 neurons = 11.17 ± 1.52 , $n = 48$ ROIs, $p < 0.0001$. (F) Schematic summarizing findings from these experiments:
 1189 hNR1 antibody bind within inhibitory synapses, preferentially onto excitatory neurons. Scale bar in A, C = 10
 1190 μm ; in D = $5 \mu\text{m}$. Error bars indicate SEM. Unpaired t-test was used to evaluate statistical significance. * $p < 0.05$,
 1191 ** $p < 0.01$, **** $p < 0.0001$.

1200 **Fig 9. Speculative model of effects of hNR1 antibody on cortical inhibitory neuron and network function.** In
1201 cortical neuronal cultures, hNR1 antibody does not affect NMDA currents **(A)** or synaptic transmission **(B)** of
1202 excitatory neurons, yet it selectively decreases NMDA currents on inhibitory cells **(C)**. Antibody binding further
1203 impairs inhibitory synaptic transmission **(D, E)** and decreases levels of GABA producing pre-synaptic proteins **(F,**
1204 **G)**, specifically in inhibitory-to-excitatory neuron synapses (yellow circle). Such reduced synaptic inhibitory
1205 output could result from altered transcriptional profiles of inhibitory neurons and/or local dysfunction of pre-
1206 synaptic NMDARs within these inhibitory synapses **(H)**, mechanisms yet to be explored. This reduced inhibitory
1207 function disinhibits activity of excitatory neurons and the network as a whole **(I, J)**.

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hippocampus - excitatory neurons

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