A novel method using ambient glutamate for the electrophysiological quantification of extrasynaptic NMDA receptor function in acute brain slices

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Key points

• We present a novel protocol to quantify extrasynaptic NMDA receptor function utilizing the semi-selective activation of extrasynaptic receptors by ambient extracellular glutamate in acute brain slices from adult rats.
• We use whole cell patch clamp to measure the effect of the NMDA receptor antagonist MK-801 on both synaptic and brief, local agonist application-evoked responses.
• The level of ambient glutamate was estimated from tonic NMDA receptor activity to be \( \approx 77 \text{ nM} \) and an equivalent concentration of NMDA was used to estimate the degree of extrasynaptic blockade (>82%) by our MK-801 protocol.
• The extrasynaptic component of the total NMDA receptor pool can be mathematically derived from these data and was estimated to be 29–39% in the stratum radiatum of the CA1 region of the rat hippocampus.
• This technique could be used to quantify extrasynaptic NMDA receptor function in rodent models of diseases where extrasynaptic NMDA receptors are implicated in neuron death.

Abstract  Synaptic NMDA receptors (NMDARs) play a central role in pro-survival signalling and synaptic plasticity in the majority of excitatory synapses in the central nervous system whereas extrasynaptic NMDARs (ES-NMDARs) activate pro-death pathways and have been implicated in many neurodegenerative diseases. ES-NMDARs have been characterized in acute brain slice preparations using the largely irreversible, activity-dependent NMDAR antagonist MK-801 to block synaptic NMDARs. This approach is limited by the concomitant MK-801 blockade of ES-NMDARs activated by ambient extracellular glutamate, which is largely absent from the synaptic cleft due to the high density of nearby glutamate transporters. In acute hippocampal slices from rats aged 35–42 postnatal days, we estimated ambient glutamate to be 72–83 nM.
resulting in a block of more than 82% of ES-NMDARs during a 5 min MK-801 application. This paper describes a novel electrophysiological and mathematical method to quantify the proportion of NMDARs located at extrasynaptic locations in a confined region of an acute brain slice preparation using MK-801 to preferentially block ES-NMDARs. The protocol uses whole cell patch clamp measurement of NMDAR responses to synaptic stimulation and brief local pressure application of NMDA before and after MK-801 application. After mathematically correcting for the relative block of both synaptic and extrasynaptic receptors, ES-NMDARs were estimated to comprise 29–39% of the total NMDAR pool in the apical dendrites of hippocampal CA1 pyramidal neurons. This new method may prove useful for accurate quantification of NMDAR distributions in neurodegenerative diseases that are associated with increased toxic ES-NMDAR signalling.

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Introduction

N-Methyl-D-aspartate (NMDA) receptors (NMDARs) are glutamate gated non-selective cation channels that can be divided by their location into synaptic and non-synaptic (termed extrasynaptic) subsets using various methodological processes (see below) (Tovar & Westbrook, 2002; Petralia et al. 2010; Gladding & Raymond, 2011). Synaptic NMDARs can play a central role in synaptic plasticity and activate gene expression programmes promoting neuronal survival and long-term neuroadaptive processes such as addiction, neurogenesis, and learning and memory (Harris et al. 1984; Bliss & Collingridge, 1993; Bading, 2013). Extrasynaptic NMDARs (ES-NMDARs) have a distinct physiological and pathophysiological function (Hardingham & Bading, 2002; Bordji et al. 2010; Kaufman et al. 2012; Oikonomou et al. 2012; Papouin et al. 2012; Wu et al. 2012). The activation of ES-NMDARs under physiological conditions is believed to arise from ‘spill-over’ of synaptic glutamate into the extrasynaptic space during intense synaptic activity (Asztely et al. 1997) which is proposed to access receptors within the proximity of the postsynaptic density (around 300 nm) termed perisynaptic NMDARs (see Kohr, 2006; Groc et al. 2009). ES-NMDARs located on the dendritic shaft may play a role in the regulation of dendritic excitability and plasticity (Kullmann et al. 1996; Clark & Cull-Candy, 2002; Losonczy et al. 2008; Wu et al. 2012).

ES-NMDARs are also activated by ambient glutamate whose levels in the extracellular space are estimated to be 25–90 nM in brain slices (Cavelier & Attwell, 2005; Herman & Jahr, 2007; Le Meur et al. 2007). Much higher ambient glutamate levels reported using microdialysis probes in vivo are likely to be an artefact of local tissue damage caused by the probe (Nyitrai et al. 2006; Sun et al. 2014). The pathological activation of ES-NMDARs by ambient glutamate in energy deprivation and conditions such as stroke (Jabaudon et al. 2000; Stanika et al. 2009; Tu et al. 2010; Suarez-Pinilla et al. 2014) is believed to arise from the reverse function of glutamate uptake by glia whose normal physiological function is to buffer extracellular glutamate and form a protective cap around the synaptic cleft (Lozovaya et al. 2004; Le Meur et al. 2007; Wu et al. 2012; Bading, 2017). The excessive activation of ES-NMDARs has also been implicated in the neurotoxicity associated with epilepsy, traumatic brain injury, stroke, Alzheimer’s and Huntington’s disease (Okamoto et al. 2009; Milnerwood et al. 2010; Frasca et al. 2011; Parsons & Raymond, 2014). Although glutamate-induced excitotoxicity has been further implicated in other disease processes such as amyotrophic lateral sclerosis, Parkinson’s disease, major depression and multiple sclerosis (Lau & Tymianski, 2010; Kim & Na, 2016; Pal, 2018), conclusive evidence is lacking partly due to difficulties in reliably assessing ES-NMDAR function. Methods to quantify ES-NMDAR numbers and functional activity and their modulation by various neuropathologies are needed to assess their involvement in various disease processes and to test effective treatment strategies to selectively reduce their neurotoxic function.

ES-NMDARs have been characterized by immuno-histochemistry and electron microscopy (Zhang & Diamond, 2006; Petralia et al. 2010) and functionally with electrophysiological (Tovar & Westbrook, 2002; Groc et al. 2007, 2009; Harris & Pettit, 2007, 2008) and calcium imaging techniques (Hardingham et al. 2002; Bengtson et al. 2008; Hardingham & Bading, 2010). These functional estimates of ES-NMDAR numbers have largely been based on the quasi-irreversible NMDAR open channel blocker, (5S,10R)-(-)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK-801) applied during synaptic stimulation to block synaptic NMDARs allowing the remaining, predominantly ES-NMDARs to be quantified usually by bath application of NMMA. Using such methods, the proportion of NMDARs comprising...
ES-NMDARs has been estimated to be approximately 75% in 1–7 days in vitro (DIV) and 19% in 7–14 DIV single cell micro-island cultures (Rosenmund et al. 1995; Tovar & Westbrook, 1999), 20–50% in standard monolayer primary culture systems (10–21 DIV) (Ivanov et al. 2006; Thomas et al. 2006; Papadia et al. 2008; Puddifoot et al. 2012), 36% in acute juvenile hippocampal slice preparations (Harris & Pettit, 2007) and 30% in acute slices from adult rats (Papouin et al. 2012). Such protocols using MK-801 to block synaptic NMDARs in acute brain slice preparations are, however, likely to underestimate the ES-NMDAR function due to the MK-801 blockade of ES-NMDARs which have been shown to be tonically active in slice preparations due to the presence of ambient glutamate (Sah et al. 1989; Cavelier & Attwell, 2005; Herman & Jahr, 2007; Le Meur et al. 2007). Evidence that ambient glutamate interacts selectively with ES-NMDARs has also prompted the use of MK-801 to selectively block the tonically active ES-NMDARs (Le Meur et al. 2007); however, this has not yet been used as a tool to estimate their relative functional proportion of the total NMDAR pool. Assessment of the blockade of both synaptic and ES-NMDAR pools during all MK-801 protocols appears necessary to generate an accurate estimate of their relative function.

The compartmentalization of the NMDA receptor endogenous co-agonists, D-serine and glycine between synaptic and extrasynaptic locations has been used to infer the relative contribution of each receptor subset to plasticity and neurotoxicity in acute slices from adult rats (Papouin et al. 2012). Similarly, memantine, a non-competitive voltage-dependent open channel blocker of NMDARs with a fast off rate, has been shown to preferentially inhibit ES-NMDARs over synaptic NMDARs with a fast off rate, which has been shown to preferentially inhibit ES-NMDARs over synaptic NMDARs with an estimated preference of 2:1 (Xia et al. 2010). However, neither co-agonist compartmentalization nor the preference of memantine for ES-NMDARs has been used to isolate or quantify the relative expression or functional proportion of synaptic/extrasynaptic NMDARs. Indeed these papers and others use a protocol based on MK-801 blockade of synaptic NMDA receptors to quantify ES-NMDAR function.

Desensitization and trafficking of NMDARs present complex obstacles to their accurate functional quantification. A rapid lateral translocation between synaptic and extrasynaptic NMDAR pools has been reported to occur in autaptic and primary cultures (Tovar & Westbrook, 2002; Groc et al. 2006, 2007). NMDAR internalization also occurs within minutes, is regulated by activity through phosphorylation–dependent mechanisms, and is primed by glycine when applied at concentrations higher than those necessary for coactivation of the NMDAR (>20 µM) and by serine (Nong et al. 2003; Imamura et al. 2008; Ferreira et al. 2017). Protocols requiring two NMDA response measurements separated by long intervals, such as is required for MK-801 application and wash out, need to verify that the second measurement is not affected by NMDAR trafficking and/or internalization. Desensitization of NMDARs occurs in a receptor subunit and voltage-dependent manner in response to internal calcium, external zinc, protons, glycine and polyamines (Nahum-Levy et al. 2001; Li et al. 2003; Cummings & Popescu, 2015). The calcium permeability of NMDARs produces a time-dependent decay in current over a few seconds, which is dependent on initial internal calcium and its buffering with endogenous and exogenous chelators. Desensitization caused by prolonged exposure to NMDA with slow agonist application systems masks a proportion of the NMDAR response and requires extended time to recover from desensitization before reapplying NMDA. Some estimates of ES-NMDAR function to date have employed bath application of NMDA (Bengtson et al. 2008; Xia et al. 2010). Other authors have used local pressure application with a picospritzer, which produces a rapid response onset but shows a prolonged decay time due to slow removal of the agonist by bath perfusion (Papouin et al. 2012).

While the use of glutamate uncaging or intense synaptic stimulation (to induce spillover of glutamate outside the synaptic cleft) achieve briefer exposure of the receptor to agonists, they activate a very small sample or a persynaptic subset of receptors which may not be representative of the entire ES-NMDAR pool. Thus, it is important to use brief infrequent agonist exposure and verify the stability of the NMDAR pools for the duration of the protocols used to quantify them.

The aim of this study was to develop an improved technique using standard patch clamp electrophysiology to characterize the proportion of the functional NMDAR pool localized to the extrasynaptic plasma membrane as a proportion of the total functional pool of NMDARs in a standard acute brain slice preparation from an adult rat. Like most other methods to quantify extrasynaptic NMDA receptor function we used the largely irreversible open channel blocker MK-801. However, our technique is novel in that it relies on ambient glutamate selectively activating ES-NMDARs for blockade with MK-801 based on the protective cap hypothesis (Lozovaya et al. 2004). We verified the presence of a tonic NMDAR current, estimated the concentration of ambient glutamate critical for the assumptions underlying our calculations, optimized our protocol to minimize artifacts from internalization, trafficking and rundown, and quantified the proportion of MK-801 blockade of synaptic and total NMDAR function. We then used these empirical measurements to mathematically estimate the proportion of ES-NMDARs in the total NMDAR pool and discuss the caveats of this estimate and the methods used.
Methods

Ethical approval

All experiments were carried out according to the German guidelines on the care and use of animals in research and with the European Community Council Directive 2010/63/EU and were approved by the local animal care committee (Regierungspräsidium, Karlsruhe, Germany; animal experimentation license T-16/19). All experiments comply with the regulations for animal usage set out by The Physiological Society.

Brain slice preparation

Whole-cell patch clamp recordings were made from CA1 pyramidal neurons in brain slices prepared from the ventral hippocampus of Sprague Dawley rats of either sex (postnatal days (P) 35–42) purchased from Charles River Laboratories (Wilmington, MA, USA; RRID:RGD_734476). Animals were housed in the specific pathogen-free animal facilities of the University of Heidelberg in 50–60% humidity at 22 ± 2°C on a 12 h/12 h dark/light cycle with ad libitum access to food and water. Polycarbonate huts were included in cages for environmental enrichment. Rats were anaesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma, St. Louis, MI, USA) by inhalation and killed by decapitation. The brain was rapidly removed and submerged in ice-cold slicing solution (in mM: sucrose, 150; NaCl, 40; KCl, 4; MgCl₂, 7; NaH₂PO₄, 1.25; CaCl₂, 0.5; glucose, 10; NaHCO₃, 26; gassed with 95% O₂ and 5% CO₂). Slices, 300 µm thick, were cut at an angle of approximately 30° above the horizontal using a vibratome in slicing solution maintained at 0°C (CU65 cooling unit and HM650V vibratome, Microm, Walldorf, Germany). Hippocampi were dissected out of each slice and transferred to a holding chamber containing aCSF (in mM: NaCl, 125; KCl, 3.5; MgCl₂, 1.3; NaH₂PO₄, 1.2; CaCl₂, 2.4; glucose, 10; NaHCO₃, 26; gassed with 95% O₂ and 5% CO₂, osmolarity 320 ± 5 mosmol l⁻¹). Slices were maintained at 32°C for the first 30 min and then returned to room temperature until used for recording over the subsequent 4 h. Parts of the dentate gyrus and the CA3 field were removed by a single diagonal cut just before a slice was transferred into the recording chamber (Otmakhova & Lisman, 1999; Otmakhova et al., 2002).

Patch clamp recordings

Single slices were transferred to a recording chamber (PM-1, Warner Instruments, Hamden, CT, USA) and completely submerged with warmed (32–34°C), continuously flowing (3–4 ml min⁻¹) aCSF. The chamber was mounted on a fixed-stage upright microscope (BX51WI, Olympus, Hamburg, Germany). Differential interference contrast optics, infrared illumination and a charged coupled device (CCD) camera (Photometrics Coolsnap HQ, Visitron Systems, Puchheim, Germany) were used to view neurons on a computer monitor using a software interface (Metamorph, Universal Imaging Systems, Downingtown, PA, USA). Patch electrodes (2–3 MΩ) were made from borosilicate glass (1.5 mm, WPI, Sarasota, FL, USA) and filled with a potassium methylsulphate-based solution (containing in mM: KMeSO₃, 131, spermine 0.6, NaCl 8, KCl 12, HEPES 10, potassium phosphocreatine 10, Mg₂-ATP 4, Na₃-GTP 0.5, Alexa-594 (Invitrogen, Waltham, MA, USA) 0.02; pH 7.35 with KOH, osmolarity 305 ± 5 mosmol l⁻¹). Recordings were made with a Multiclip 700A or 700B amplifier, digitized through a Digidata 1322A A/D converter, acquired and analysed using pCLAMP 9 software (Axon Instruments and Molecular Devices, Union City, CA, USA). Cells were identified as pyramidal by their action potential waveform showing slow firing with large after-hyperpolarization potentials in response to current injection. Access (range: 10–22 MΩ) and membrane resistance (range 60–140 MΩ) were monitored regularly during voltage clamp recordings and data were rejected if changes in access resistance greater than 25% occurred. NMDAR-mediated currents were recorded in low Mg²⁺ (0.1 mM) aCSF containing glycine (20 µM), picrotoxin (100 µM, Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 µM, Tocris) or 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 µM, Tocris), strychnine (1 µM, Sigma) at a holding potential of −71 mV. All membrane potentials have been corrected for the calculated junction potential of −11 mV (JPCalc program by Dr Peter H. Barry, UNSW Sydney, Australia). Slices were incubated in MK-801 (20 µM, Tocris, Bristol, UK) where indicated. EPSCs were evoked in response to single 100 µs constant current stimulus (80–200 µA) from an A365 stimulus isolator (World Precision Instruments (WPI), Sarasota, FL, USA). NMDA (1–2 mM) was applied from a patch pipette (1–2 µm opening) positioned near the field stimulator and connected to a picospritzer (PV800, WPI; 5–10 p.s.i. for 100–600 ms). Suction pipettes were formed from a broken patch electrode, fire-polished to a 6–8 µm tip diameter, connected to a suction system of the picospritzer, positioned around 100 µm from the tip of the puffer pipette and activated for 5 s from a time point 1 s before each NMDA puff application.

Statistics and data analysis

Data are presented as mean with standard deviation (SD) in the Results text and figures. In total, 49 animals were killed during the study. Only one recording per slice was performed. The number of cells (n) and the number of animals (N) for each dataset is indicated in the Results
section and figure legends. A skewness/kurtosis test for normality was performed on all data and the hypothesis that the data were normally distributed could not be rejected in all cases. Hence, a normal distribution was assumed and statistical comparisons were made using cell numbers as the experimental unit and using two-tailed Student's t-tests for independent samples, unless otherwise stated. Statistical evaluation and graph-plotting were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) and Stata Statistical Software (StataCorp. 2013, College Station, TX, USA). For data analysis, Excel 2010 (Microsoft, USA) and Clampfit (Axon Instruments and Molecular Devices, Union City, CA, USA) were used.

**Abbreviations**

cSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APV, 2-amino-5-phosphonovaleric acid; CA1, cornu ammonis area 1; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days in vitro; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EPSC, excitatory postsynaptic current; ES-NMDARs, extrasynaptic NMDARs; MK-801, (5S,10R)-(+-) -5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate; NMDA, N-methyl-D-aspartate; NMDARs, NMDA receptors; SD, standard deviation; SYN-NMDARs, synaptic NMDARs.

**Results**

Our experimental approach to quantify the extrasynaptic proportion of the total functional pool of NMDARs in the proximal apical dendrite of the CA1 pyramidal neuron in a standard acute brain slice preparation from an adult rat was to quantify responses to exogenous NMDA before and after a relatively selective ES-NMDAR blockade with a protocol which employs the activity-dependent, open channel blocker MK-801. Our approach assumes neither a complete blockade of ES-NMDARs nor the absence of any synaptic NMDAR blockade by MK-801 but instead measures the blockade of synaptic and total NMDAR pools to mathematically generate an estimate of the ES-NMDAR pool.

**Verification of ambient glutamate activation of ES-NMDARs**

Our method to quantify ES-NMDAR function utilizes the quasi-irreversible, activity-dependent open channel blocker, MK-801, applied in basal unstimulated conditions at hyperpolarized membrane potentials to preferentially block ES-NMDARs due to their tonic activity in acute slice preparations. MK-801 can freely enter the synapse and will block to some extent spontaneously active synapses in addition to ES-NMDARs tonically activated by ambient glutamate. Such tonic NMDAR currents have been repeatedly demonstrated in acute slices and are believed to arise from ambient extracellular glutamate activation of ES-NMDARs (Herman & Jahr, 2007; Le Meur et al. 2007). Selective interaction of ambient glutamate with ES-NMDARs arises due to a protective cap of glutamate transporters located primarily on astrocytes surrounding synapses which prevents ambient glutamate from entering the synaptic cleft and compartmentalizes glutamate between synaptic and extrasynaptic locations (Lozovaya et al. 2004; Herman & Jahr, 2007; Le Meur et al. 2007; Wu et al. 2012) (Fig. 1). To verify the presence of ambient glutamate in our experimental settings, we applied either APV (50 µM) or MK-801 (20 µM) to CA1 pyramidal neurons voltage clamped at −71 mV in solutions containing reduced Mg2+ concentrations (0.1 mM) to relieve the Mg2+ block and unmask NMDAR currents at hyperpolarized potentials. As expected, these NMDAR antagonists produced a small outward current (MK-801: 19.5 pA, SD = 10.7 pA; n = 19, N = 13; APV: 21.4 pA, SD = 9.0 pA; n = 5, N = 4) indicative of tonic NMDAR activation which was larger than current changes measured under identical control conditions in the absence of NMDAR blockers (−3 pA, SD = 6.5 pA; n = 9, N = 8) (Fig. 2B). This outward current induced by NMDAR antagonists reflects the presence of tonic NMDAR activity in CA1 pyramidal neurons of our hippocampal slices with a conductance of 0.27 nS, SD = 0.12 nS. This indicates a low concentration of ambient glutamate, which we next attempted to quantitatively estimate.

To assess the ambient glutamate concentration in our experimental system we used an approach published by Cavelier & Attwell (2005). A saturating concentration of NMDA (200 µM) activated a peak current of 3.7 nA (SD = 0.8 nA, n = 5, N = 4) which decreased to a slowly declining plateau current of 2.6 nA, SD = 1.1 nA after 1.5 min following receptor desensitization (Fig. 2C). Thus, the tonic current of 21.4 pA which is unmasked by a saturating concentration of APV (Fig. 2B) and activated by the ambient glutamate represents 0.8% of the desensitized current produced by prolonged exposure to a saturating NMDA concentration. Taking into account that glutamate produces a maximum current that is 1.2- to 1.5-fold larger than that produced by NMDA (Priestley et al. 1995) we can estimate that tonic currents represent the activation of 0.55–0.69% of the total NMDAR pool. Ambient glutamate concentration ([glu]) can be calculated from the Hill equation using previous estimates of the Hill coefficient (nH = 1.5) and half-maximal effective concentration (EC50 = 2.3 µM) (Patneau & Mayer, 1990).

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\text{response to } [\text{glu}] \text{ maximum response} = \frac{1}{1 + \left( \frac{\text{EC}_{50}}{[\text{glu}]^n} \right)} \tag{1}
\]
According to eqn (1), ambient glutamate concentration lies between 72 nM and 83 nM, which is in line with the results presented by Cavelier and Attwell (2005) of 77–89 nM at 35°C.

The presence of ambient extracellular glutamate presumably causes the stochastic opening, in the virtual absence of extracellular Mg²⁺, of a small percentage of extrasynaptic NMDA receptor-associated channels at any given moment in time which will be blocked in a cumulative manner over time by the largely irreversible open channel blocker MK-801. The maximum effect of MK-801 to cause an outward current occurred in 4.93 ± 0.38 min at which point we can assume that the vast majority of ES-NMDARs are irreversibly blocked. For this reason, we chose an incubation period of 5 min for our MK-801 incubation to block the vast majority of ES-NMDARs for our protocol to estimate ES-NMDAR function (see below).

Empirical measurement of synaptic and total NMDAR function

Synaptic NMDAR function is typically defined by responses to low-frequency (≤0.1 Hz) single presynaptic stimulations using a field stimulator to activate axon terminals within a limited radius of the stimulator whose extent is dependent upon the stimulation intensity. Estimates of the total NMDAR pool in a portion of the dendritic tree were made with brief local pressure applications of NMDA near the field stimulator. To further confine the spatial and temporal window of NMDAR activation, we developed a combined ‘puff–suction’ method by adding a local suction pipette for rapid removal of NMDA (see Methods section). This puff–suction method enabled a brief agonist exposure to a restricted area of the dendritic tree (Fig. 3; Video S1 in the online Supporting information). Current responses showed a rise...
time (10–90% of the peak) of 0.36 s, SD = 0.1 s; n = 8, N = 7 and a decay time (90–10% of the peak) of 0.7 s, SD = 0.4 s; n = 8, N = 7 which is dramatically faster than puff responses without suction (rise time of 8.9 s, SD = 5.5 s; decay time 7.52 s, SD = 1.6 s; n = 6, N = 5; Fig. 3B). This puff–suction technique reliably produced stable response amplitudes over recordings lasting more than 1 h without rundown (Fig. 4). This is a simple and inexpensive semi-rapid application system to evoke repeated NMDAR-mediated responses in a restricted region of the dendritic field.

Validation of the ES-NMDAR blockade protocol

NMDAR desensitization, trafficking and internalization must be avoided to assess the effects of MK-801 on NMDAR responses. In order to verify the stability of our responses over the time frame of the experiments we recorded synaptic and puff–suction responses before and after a 30 min pause during which MK-801 bath application and its wash out would normally take place (Fig. 4A). Excitatory postsynaptic currents (EPSCs) evoked with 100 µs unipolar pulses at 0.1 Hz produced a stable average amplitude (68.9 pA, SD = 21.2 pA; n = 10, N = 8) over a 10 min baseline recording which was not altered after a 30 min pause in the recording (100.2%, SD = 14.5% of baseline; n = 10, N = 8; Fig. 4B and C). Similarly, puff–suction responses recorded at 60 s intervals were stable in their amplitude over five responses in baseline (217.0 pA, SD = 99.0 pA; n = 10, N = 8).

The first puff response after the 30 min break showed an increased amplitude (128.5%, SD = 88.9% of baseline; n = 12, N = 10; Fig. 4B); however, the following four puff–suction responses did not differ from baseline responses in their amplitudes (101.4%, SD = 17.6% of baseline; n = 10, N = 8, Fig. 4B and C). In light of these results we decided to use the second to fifth puffs after the wash out of MK-801 for the quantification of total NMDAR function.

Estimation of the proportion of ES-NMDARs in the apical dendrites

Having defined and optimized the methods to measure synaptic and total NMDAR pools in a restricted segment of the CA1 pyramidal cell dendritic field with minimal rundown artifacts, we next measured these responses before and after the application of MK-801. Synaptic NMDAR currents and NMDAR currents elicited by puff application were recorded before and after a 5 min application of MK-801 (20 µM) and its subsequent wash out (25 min) at a holding potential of −71 mV in 0.1 mM Mg2+ (Fig. 5A). MK-801 reduced the amplitude of both EPSC and puff–suction responses (EPSC: 75.9%, SD = 18.5%, n = 9, N = 7; puff: 52.6%, SD = 18.2%, n = 9, N = 7, of their mean baseline responses, respectively, Fig. 5B, C and D). The significantly greater blockade of puff responses by MK-801 (P = 0.016) indicates that our puff–suction responses arise in part from non-synaptic NMDARs and is in line with our assumption that MK-801

Figure 2. Tonic NMDAR activity indicative of ambient extracellular glutamate is unmasked by NMDAR antagonists

A, a representative recording of the holding current change induced by MK-801 at a holding potential of −71 mV. B, the histogram shows the mean ± SD change in holding current induced by bath application of MK-801 (20 µM) in aCSF containing 0.1 mM Mg2+ (n = 19, N = 13) and 0 mM Mg2+ (n = 10, N = 8), or APV (50 µM) in aCSF containing 0.1 mM Mg (n = 5, N = 4) or aCSF containing 0.1 mM Mg without an NMDAR antagonist (control, n = 9, N = 8). C, summary histogram shows the mean (±SD) of the response to a saturating concentration of NMDA (200 µM) measured at its peak (black column) and 1.5 min later in its desensitized steady state (red column) (n = 5, N = 4). [Colour figure can be viewed at wileyonlinelibrary.com]
preferentially blocks the ES-NMDAR pool due to its tonic activation by ambient glutamate (see Fig. 1). In addition, we analysed the rise and decay kinetics of both synaptic and puff responses and found no effect of MK-801 on these properties (Fig. 5E and F). The rapid EPSC decay time of ~24 ms is consistent with predominantly GluN2A-containing synaptic receptors recorded at physiological temperatures at the maturational state of P35–42 in rats (Stocca & Vicini, 1998; Vicini et al. 1998; Korinek et al. 2010; Paoletti et al. 2013; Wyllie et al. 2013).

**No recovery of the EPSCs or puff responses after wash out of MK-801**

An incomplete wash out of MK-801 or significant trafficking of NMDARs between synaptic, extrasynaptic and intracellular compartments during this experiment would affect our synaptic and puff responses and violate our experimental assumptions. To further test the stability of the synaptic and ES-NMDAR pools in our protocol we recorded EPSC and NMDA puff–suction responses for longer periods following MK-801 wash out in a subset of cells. Analysis of the amplitude of EPSCs and puff–suction responses recorded for 10 min after the application and wash out of MK-801 showed a remarkable stability (Fig. 6). The amplitudes of the last five EPSCs of a 10 min recording period after MK-801 wash out accurately reflect the average of the entire 10 min period (Fig. 6A). The average of the last five EPSCs in a post-MK-801 recording (96.1%, SD = 9.3%; n = 4, N = 4) did not significantly differ from the last five EPSCs during a pre-MK-801 assessment (100.1%, SD = 9.4%; n = 9, N = 7, P = 0.43). The stability of post-MK-801 EPSCs indicates that no measurable recovery of the synaptic NMDAR pool due to trafficking of unblocked NMDARs into the synapse and/or MK-801 unblock. In addition, the puff–suction response, was stable over a 10 min post-MK-801 wash out recording (amplitude last/first response = 97.8%, SD = 18.4%; n = 9, N = 7, P = 0.68, paired t-test, Fig. 6B). These results indicate that the 25 min wash out period of MK-801 was sufficient to recommence stimulation without causing further MK-801 blockade and that the

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**Figure 3. The puff–suction technique for the analysis of NMDAR function**

*A*, the arrangement of the pipettes for patch clamp, electrical stimulation, pressure application of NMDA (puff) and suction-based removal of NMDA (suction) is shown schematically in an apical dendrite. *B*, the histogram shows the mean (±SD) of the rise and decay time (from 10 to 90% of response peak) of the puff responses with or without suction. *C* and *D*, representative responses to puff application of NMDA (1 mM, 5 p.s.i., for 300 ms) without (*C*) and with (*D*) suction. Upper panels show membrane current responses (black trace before MK-801 application, red trace after 30 min wash out). Lower panels show visual representation of the maximum puff area. The puffer pipette (puff) as well as the patch pipette (patch) were filled with the fluorescent dye Alexa 594. The proximal apical dendrite (arrowhead) is also visible. Scale bar, 50 µm. Heat map: white, maximal dye concentration; purple, no dye. See also Video S1 in the online Supporting information. [Colour figure can be viewed at wileyonlinelibrary.com]
synaptic and total NMDAR receptor number remained stable over the time frame of the experiment. This suggests that our protocol did not suffer from response rundown due to, e.g., NMDAR internalization, nor from response recovery due to, e.g., trafficking or MK-801 unblock.

**Derivation of equations to estimate the proportion of ES-NMDARs in the total NMDAR pool**

Having completed empirical functional measurements of a sample of the synaptic and total NMDAR pool before and after MK-801 application, we wished to mathematically estimate the proportion of the NMDAR pool which is extrasynaptic. We began by mathematically defining the relevant parameters. Whether considering the entire patched neuron or a subregion of its apical or basal dendrites, the total pool of functional NMDARs can be assumed to be composed of two mutually exclusive groups, ES-NMDARs (ES\textsubscript{NMDAR}) and synaptic NMDARs (SYN\textsubscript{NMDAR}).

\[
\text{TOTAL}_{\text{NMDAR}} = \text{ES}_{\text{NMDAR}} + \text{SYN}_{\text{NMDAR}}
\]

(2)

The puffer is assumed to activate a spatially restricted subgroup of the total NMDAR pool that is composed of both ES-NMDARs and synaptic NMDARs. Thus PUFF\textsubscript{beforeMK} is a sample of TOTAL\textsubscript{NMDAR} with the puff response being composed of synaptic (SYN) and extrasynaptic (ES) components (Fig. 1B).

\[
\text{PUFF}_{\text{beforeMK}} = \text{ES}_{\text{beforeMK}} + \text{SYN}_{\text{beforeMK}}
\]

(3)

Similarly we can divide the puff response after MK-801 application into its synaptic and ES-NMDAR components.

\[
\text{PUFF}_{\text{afterMK}} = \text{ES}_{\text{afterMK}} + \text{SYN}_{\text{afterMK}}
\]

(4)

---

**Figure 4. EPSC and puff responses remain stable for duration of the protocol**

**A**, above: schematic representation of the protocol of the control experiments to identify any changes in synaptic or puff responses independent of MK-801 application. Below: a representative recording from a control experiment. All recordings were made at a holding potential of $-71$ mV in 0.1 mM Mg$^{2+}$ in the presence of 20 $\mu$M glycine and blockers of AMPA, GABA\textsubscript{A} and glycine receptors (see Methods). Blue arrows indicate EPSCs and red arrows puff responses in the representative recording. 

**B**, the synaptic and puff responses do not change after a 30 min interval equivalent to that required for MK-801 application and wash out. The graph shows the mean ± SD synaptic ($n = 10, N = 8$) and puff ($n = 10, N = 8$) responses for the experimental protocol shown in A. Response data are normalized to the response average measured in baseline before the 30 min pause. For illustration purposes, only every fifth EPSC data point before the break and every second EPSC after the break is plotted. 

**C**, summary histogram shows the mean (±SD) of all NMDA puff and NMDAR EPSC response amplitudes after the 30 min break normalized to the mean responses in the baseline recordings before the break. The first puff response after the break was excluded from this analysis. Neither puff nor EPSC responses were altered by the 30 min break in the recording (puff: 101.4%, SD = 17.6% EPSC: 100.2%, SD = 14.5%). 

**D**, representative traces of puff (upper panel) and EPSC (lower panel) responses before (black) and after (red) the 30 min break. Traces represent the average of four puff and five EPSC responses. [Colour figure can be viewed at wileyonlinelibrary.com]

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MK-801 application is designed to block the majority of ES-NMDARs on the patched neuron and is likely to block some proportion of synaptic NMDARs that are activated by the spontaneous release of glutamate at synapses. If MK-801 were to block 100% of ES-NMDARs then $E_{\text{afterMK}} = 0$. Thus, eqn (4) becomes:

$$\text{PUFF}_{\text{afterMK}} = \text{SYN}_{\text{afterMK}}$$  \hspace{1cm} (5)

We reasoned that the electrical stimulator activates a spatially restricted sample of only synaptic NMDARs that serves as an empirical estimate of $\text{SYN}_{\text{NMDAR}}$. Although we positioned our puffer and stimulator to activate as much as possible the same region of the apical dendrite it is likely that the synaptic receptor pools sampled by our puffer and stimulator are not 100% identical. Blockade of synaptic NMDARs by MK-801 application is

---

**Figure 5. The extrasynaptic block protocol in CA1 apical dendrites**

A, above: the extrasynaptic block protocol is shown schematically. Below: a representative recording of the experiment. B, summary data for the effects of the extrasynaptic block protocol on synaptic and puff responses in the apical dendrites of CA1 pyramidal cells. Plotted are the mean (±SD) EPSC and puff responses normalized to their respective baseline responses before MK-801 application for the protocol shown in A ($n = 9$ cells, $N = 7$ animals). C, summary histogram shows the mean (±SD) of all the normalized puff and EPSC response amplitudes after the MK-801 wash out. The first puff response after break is excluded from this analysis ($n = 9$ cells, $N = 7$ animals; $^*P = 0.016$ for the comparison between EPSCs and puff responses). D, representative traces of puff (upper panel) and EPSC (lower panel) responses before (black) and after (red) the MK-801 application and wash out. Traces represent the average of four puff and five EPSC responses. E and F, the kinetics of NMDAR EPSCs ($E$) and puff responses ($F$) before and after MK-801 application did not differ (EPSC rise times: $P = 0.22$, $n = 8$, $N = 7$; EPSC decay times: $P = 0.96$, $n = 8$, $N = 7$; puff response rise times: $P = 0.48$, $n = 7$, $N = 6$; puff response decay times: $P = 0.19$, $n = 7$, $N = 6$; paired $t$ tests). Rise times represent the time between 10% and 90% of the peak amplitude. Decay times represent the weighted $\tau$ (see Stocca & Vicini, 1998). [Colour figure can be viewed at wileyonlinelibrary.com]
presumably dependent on the spontaneous release of synaptic glutamate and can be assumed to be homogeneous and thus equivalent in the synapses activated by our electrical stimulator and the NMDA puff application. Thus:

\[
\frac{\text{SYN}_{\text{after} MK}}{\text{SYN}_{\text{before} MK}} = \frac{\text{EPSC}_{\text{after} MK}}{\text{EPSC}_{\text{before} MK}}
\]

(6)

\[
\text{SYN}_{\text{after} MK} = \frac{\text{EPSC}_{\text{after} MK}}{\text{EPSC}_{\text{before} MK}} \times \text{SYN}_{\text{before} MK}
\]

(7)

Substituting eqn (7) into eqn (5):

\[
\text{PUFF}_{\text{after} MK} = \frac{\text{EPSC}_{\text{after} MK}}{\text{EPSC}_{\text{before} MK}} \times \text{SYN}_{\text{before} MK}
\]

(8)

Substituting eqn (3) into eqn (8):

\[
\text{PUFF}_{\text{after} MK} = \frac{\text{EPSC}_{\text{after} MK}}{\text{EPSC}_{\text{before} MK}} \times (\text{PUFF}_{\text{before} MK} - \text{ES}_{\text{before} MK})
\]

(9)

Rearranging eqn (9) gives:

\[
\text{ES}_{\text{before} MK} = \text{PUFF}_{\text{before} MK} - \frac{\text{EPSC}_{\text{before} MK}}{\text{EPSC}_{\text{after} MK}} \times \text{PUFF}_{\text{after} MK}
\]

(10)

The estimate of the proportion of the total NMDAR pool consisting of ES-NMDARs is based on the proportion of the puff response before MK-801 application arising from ES-NMDARs:

\[
\frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL-NMDAR}} = \frac{\text{ES}_{\text{before} MK}}{\text{PUFF}_{\text{before} MK}}
\]

(11)

This ratio can be generated by dividing both sides of eqn (10) by \(\text{PUFF}_{\text{before} MK}\):

\[
\frac{\text{ES}_{\text{before} MK}}{\text{PUFF}_{\text{before} MK}} = 1 - \frac{\text{EPSC}_{\text{before} MK}}{\text{EPSC}_{\text{after} MK}} \times \frac{\text{PUFF}_{\text{after} MK}}{\text{PUFF}_{\text{before} MK}}
\]

(12)

**Figure 6.** NMDAR EPSC amplitudes remain stable both before MK-801 application and after its washout

A, plot shows the mean (±SD) EPSC amplitude over a 10 min period before MK-801 application (n = 9 cells, N = 7 animals) and after its wash out (n = 4 cells, N = 4 animals). Data are normalized to their respective mean amplitude over the entire 10 min period indicated by the dashed line at 100%. B, plotted are the mean (±SD) puff response amplitudes recorded over 10 min following the 25 min MK-801 wash out period normalized to the mean amplitude over the 10 min period (n = 9, N = 7). [Colour figure can be viewed at wileyonlinelibrary.com]
By substituting eqn (11) into eqn (12), an equation comprising experimentally measurable parameters is generated to quantify ES-NMDARs as a proportion of the total NMDAR pool:

$$\frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}} = 1 - \frac{\text{EPSC}_{\text{beforeMK}}}{\text{EPSC}_{\text{afterMK}}} \times \frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

(13)

Equation (13) (also referred to as the Café Botanik equation) was used to generate estimates of the functional ES-NMDAR pool as a percentage of the total functional NMDAR population for each cell if all ES-NMDARs are blocked by the MK-801 protocol.

**Calculation of the ES-NMDAR pool in the proximal apical dendrites of CA1 pyramidal cells**

To quantify the proportion of ES-NMDARs, eqn (13) was applied.

$$\frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}} = 1 - \frac{\text{EPSC}_{\text{beforeMK}}}{\text{EPSC}_{\text{afterMK}}} \times \frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

$$\frac{\text{EPSC}_{\text{beforeMK}}}{\text{EPSC}_{\text{afterMK}}} = 1.39, \text{ SD} = 0.36 \ (n = 9, \ N = 7)$$

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} = 0.53, \text{ SD} = 0.18 \ (n = 9, \ N = 7)$$

$$\frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}} = 0.29, \text{ SD} = 0.23 \ (n = 9, \ N = 7)$$

Thus, we conclude that ES-NMDARs compose 29% of the total NMDAR population in the apical dendrites of CA1 pyramidal cells, measured approximately 150 µm away from the soma (Fig. 3C). Note that this estimate assumes that 100% of ES-NMDARs are blocked by MK-801 and thus will underestimate the proportion of NMDARs if the MK-801 application did not achieve a 100% block of the ES-NMDARs. We next derived an equation in order to account for a possible error due to incomplete block of all ES-NMDARs.

**Correcting the estimate of ES-NMDARs for an incomplete block by MK-801**

We next attempted to model the effect of an incomplete block of ES-NMDARs by MK-801 to correct the Café Botanik eqn (13). Let the variable $X$ define the proportion of ES-NMDARs not blocked by MK-801:

$$X = \frac{\text{ES}_{\text{afterMK}}}{\text{ES}_{\text{beforeMK}}}$$

(14)

If we then substitute eqn (14) into eqn (4)

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} = X \times \frac{\text{ES}_{\text{beforeMK}} + \text{SYN}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

(15)

and insert eqn (3) into eqn (15)

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} = X \times (\frac{\text{PUFF}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}} - \frac{\text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}}) + \frac{\text{SYN}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

(16)

dividing eqn (16) by $\text{PUFF}_{\text{beforeMK}}$

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} = \frac{X}{\text{PUFF}_{\text{beforeMK}}} - \frac{X \times \text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}} + \frac{\text{SYN}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

(17)

rearranging eqn (6)

$$\frac{\text{SYN}_{\text{afterMK}}}{\text{SYN}_{\text{beforeMK}}} = \frac{\text{EPSC}_{\text{afterMK}}}{\text{EPSC}_{\text{beforeMK}}}$$

(18)

we insert eqn (18) into eqn (17)

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} = \frac{X}{\text{PUFF}_{\text{beforeMK}}} - \frac{X \times \text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}} + \frac{\text{EPSC}_{\text{afterMK}}}{\text{EPSC}_{\text{beforeMK}}} \times \frac{\text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

(19)

we subtract $X$ and place $\frac{\text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}}$ outside of the brackets

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} - X = \frac{\text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}} \times \left( \frac{\text{EPSC}_{\text{afterMK}}}{\text{EPSC}_{\text{beforeMK}}} - X \right)$$

(20)

rearranging eqn (20) gives

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} - X = \frac{\text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}}$$

(21)

we conclude as previously derived in eqns (2) and (3) that

$$\frac{\text{SYN}_{\text{beforeMK}}}{\text{PUFF}_{\text{beforeMK}}} = \frac{\text{SYN}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}} = 1 - \frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}}$$

(22)

inserting eqn (22) into eqn (21)

$$\frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} - X = 1 - \frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}}$$

(23)

rearranging eqn (22) gives

$$\frac{\text{ES}_{\text{NMDAR}}}{\text{TOTAL}_{\text{NMDAR}}} = 1 - \frac{\text{PUFF}_{\text{afterMK}}}{\text{PUFF}_{\text{beforeMK}}} - X$$

(24)

The eqn (24) is extended by the $- X$ in the numerator and the denominator in comparison to the simplified eqn (13) in the Results section. To calculate the...
ES-NMDAR proportion of the total receptor number we put the experimentally known values into eqn (25).

\[
\frac{\text{ES-NMDAR}}{\text{TOTAL NMDAR}} = 1 - \frac{0.53 - X}{0.76 - X}
\]  

(25)

This relationship is shown in Fig. 7A and depicts the proportion of ES-NMDARs in our rat brain slice preparation relative to the proportion of ES-NMDARs blocked during our MK-801 protocol.

**Estimating the ES-NMDAR blockade by our 5 min MK-801 application**

To improve the precision of our estimate of ES-NMDAR function, we need to measure the proportion of ES-NMDARs blocked by our MK-801 protocol. Since ES-NMDAR function is not directly measurable, we measured the effects of our 5 min MK-801 application on synaptic NMDAR function, which is directly measurable.

Since NMDA but not ambient glutamate can freely enter the synapse we used a low concentration of bath applied NMDA estimated to cause a similar activation of synaptic NMDARs to that caused by ambient glutamate at ES-NMDARs. Thus we used bath applied NMDA and synaptic NMDARs as a proxy for ambient glutamate and ES-NMDARs to measure the blockade caused by a 5 min MK-801 application (Fig. 7B).

To calculate an NMDA concentration with equivalent activity at NMDARs to our estimated ambient glutamate concentration of 72–83 nM (see above) we corrected for a 20.8-fold higher affinity and 1.2- to 1.5-fold higher efficacy of glutamate compared to NMDA (Lester & Jahr, 1992; Priestley et al., 1995; Herman & Jahr, 2007). Thus the equivalent NMDA concentration would be 2.07–2.25 µM.

As depicted in Fig. 7B, this experiment was identical to the MK-801 blocking protocol used in our paper except that 2 µM NMDA was co-applied with the MK-801.
NMDAR-mediated EPSCs were recorded before and after, but not during, this NMDA/MK-801 treatment or the 25 min wash out period. NMDA/MK-801 co-application blocked 82%, SD = 10.0% (n = 6, N = 4) of the synaptic NMDAR-mediated EPSCs (Fig. 7C and D). This result indicates that a 5 min MK-801 application in the presence of ambient glutamate can be expected to block 82% of ES-NMDARs. Application of 1 μM NMDA results in a blockade of 70%, SD = 14.4% (n = 8 N = 5; P = 0.11) of the EPSC amplitude (Fig. 7D). The calculated 2.25 μM NMDA concentration equivalent to our 72–83 nM estimate of ambient glutamate is likely to block more than this 82% of NMDARs. Inserting this 82% experimental estimation of ES-NMDAR blockade into eqn (24) generates an estimate of 39% as the maximum proportion of ES-NMDARs in the total NMDAR pool. This new result allows us to conclude that between 29% and 39% of the NMDA receptor pool in CA1 apical dendrites are located extrasynaptically.

Discussion

This study presents an improved protocol for assessing ES-NMDAR function in an acute slice preparation from a mature animal and used it to estimate that 29–39% of NMDARs on the apical dendrite of CA1 pyramidal cells are located extrasynaptically in acute slices from P35–42 rats recorded at 32–34°C. As for previously published methods we used the virtually irreversible NMDAR blocker MK-801 to dissect synaptic from extrasynaptic receptor pools, but our method is novel in that it uses the verified semi-selective activation of ES-NMDARs by ambient glutamate, estimated to be 72–83 nM in our slices, to preferentially block this receptor pool. The presence of a tonic NMDA receptor-mediated current and a preferential MK-801 block of agonist responses over synaptic responses supports the hypothesis that ambient glutamate is present and largely excluded from the synapse (Lozovaya et al. 2004; Le Meur et al. 2007; Wu et al. 2012) and is a prerequisite for our method. Since the compartmentalization of synapses by astrocytes is brain region dependent, this approach is not applicable in other brain regions without compartmentalization such as the nucleus accumbens (Chiu & Jahr, 2017). When applying our methodology to another cell type or brain region, functional assessment of the protective cap phenomenon according to Lozovaya et al. (2004) might be performed to verify its presence as well as the presence of ambient glutamate. Our protocol is designed to detect alterations in the relative proportion of NMDARs located at extrasynaptic sites. Any ex vivo manipulation or in vivo alterations that severely compromise glutamate transporter function and abolish compartmentalization of glutamate between synaptic and extrasynaptic spaces violate our assumptions and invalidate our protocol. Our estimate of ES-NMDAR function includes a mathematical correction for the concomitant partial block of synaptic NMDARs which can be directly measured. We also indirectly estimated the extent to which MK-801 blocked ES-NMDARs, using NMDA bath application and synaptic receptors as a proxy for ambient glutamate and ES-NMDARs, and generated a mathematical function to correct for any incomplete ES-NMDAR block in the protocol. Methods to quantify ES-NMDAR function by actively blocking synaptic NMDARs with MK-801 cannot correct for the unavoidable block of ES-NMDARs activated by ambient glutamate. Although ambient glutamate can be minimized by using cell culture or juvenile animals (see below), acute slices from adult animals are more relevant for many physiological and pathophysiological models. Our ES-NMDAR block protocol does not require perfect overlap in the synaptic and extrasynaptic NMDAR pools sampled by our NMDA puff and electrical stimulator. If synaptic stimulation is used to actively block synaptic NMDARs with MK-801 then these synaptic NMDARs must completely include those activated by exogenous agonist application or else the residual unblocked synaptic NMDARs will contribute to the estimate of ES-NMDAR function. Thus, any differences in the dendritic areas activated by the puffer and stimulator compromise such synaptic block protocols but not our ES-NMDAR block method. Although our technique is not without assumptions, it acknowledges the non-selective block by MK-801 of extrasynaptic NMDARs, which has been shown here and elsewhere to occur in slice preparations especially from adult rodents recorded at physiological temperatures. Thus, our method overcomes a major confound of MK-801 protocols to estimate ES-NMDAR function from mature animals suitable for investigating excitotoxic mechanisms in animal models of disease.

That said, our method generated an estimate of the ES-NMDAR pool remarkably similar to those of two previous studies of the apical dendrites of hippocampal pyramidal cells in acute slices, which were 36% (Harris & Pettit, 2007) and 30% (Papouin et al. 2012). Despite this similarity, major differences in animal age and methodology limit comparisons with our results. The use of younger rats and lower recording temperatures by Harris and Pettit (P14–22, room temperature; our study: P35–42, 34°C) likely results in less MK-801 blockade of ES-NMDARs due to lower concentrations of ambient glutamate. Ambient glutamate concentration depends partly on animal age, slice thickness, recording depth and recording temperature (Sah et al. 1989; Le Meur et al. 2007) and may be altered by toxic insults or in slices from disease model animals. Ambient glutamate concentrations are estimated to be 27–33 nM at 24°C in contrast to 72–89 nM at 35°C (Cavelier & Attwell, 2005) and 72–83 nM at 34°C (our study). Brain glutamate concentrations in vivo have been shown to double between P14 and P21 (Tkac et al. 2012).
NMDAR pools have also been reported to be stable in during the post MK-801 wash out indicates that no lateral experiments. In addition, the lack of recovery of EPSCs of NMDARs into the membrane during the course of our suggests there is no measurable insertion or internalization recovery in the puff response after MK-801 application to produce stable response amplitudes over recordings a simpler and less expensive alternative, which we found dendritic regions and the latter requires expensive imaging techniques can also generate a rapid response onset from rapid piezo application systems or glutamate uncaging quantified with minimal receptor desensitization. While protocol to allow the total NMDAR function to be clearly demonstrate the need to correct for this synaptic NMDAR function in acute slice preparations. The greater reduction by MK-801 of the puff response compared to the EPSC amplitude is consistent with the assumption that ES-NMDARs exist and are preferentially activated by ambient glutamate. The higher glutamate affinity, slower deactivation and longer open times of GluN2B-containing NMDARs (Wyllie et al. 2013) may increase their susceptibility to MK-801 blockade thus contributing to its preferential effect at ES-NMDARs which are predominantly GluN2B-containing NMDARs in adult rodents (Tovar & Westbrook, 1999; Groc et al. 2006; Harris & Pettit, 2007; Petralia et al. 2010). Our reduction in EPSC amplitude of 24.1% resembles the results of Scimemi et al. (2004) who showed a 28% decrease after a 15 min 4 µM MK-801 application. The reduction in the EPSC amplitude after MK801 application is most likely due to spontaneous synaptic NMDAR activation resulting from spontaneous synaptic glutamate release and/or ambient extracellular glutamate entering the synaptic cleft. These results clearly demonstrate the need to correct for this synaptic NMDAR block when estimating ES-NMDAR function from MK-801 protocols in acute slice preparations.

The rapid response onset and removal of NMDA with our puff–suction technique was critical for this protocol to allow the total NMDAR function to be quantified with minimal receptor desensitization. While rapid piezo application systems or glutamate uncaging techniques can also generate a rapid response onset from a brief agonist pulse, the former cannot sample restricted dendritic regions and the latter requires expensive imaging equipment. The puff–suction technique developed here is a simpler and less expensive alternative, which we found to produce stable response amplitudes over recordings lasting more than 1 h.

 Trafficking of NMDARs would alter the estimation of different NMDAR pools enormously. The absence of any recovery in the puff response after MK-801 application suggests there is no measurable insertion or internalization of NMDARs into the membrane during the course of our experiments. In addition, the lack of recovery of EPSCs during the post MK-801 wash out indicates that no lateral translocation is detectable in our experimental settings. NMDAR pools have also been reported to be stable in juvenile (P14–22 rat) acute brain slices (Harris & Pettit, 2007) and at postsynaptic but not autaptic presynaptic NMDARs in P21–35 rat brain slices (Yang et al. 2008). However, a rapid exchange of synaptic and ES-NMDAR pools occurs in slices from neonatal (P2–9) rats (Bellone & Nicoll, 2007) and immature (6–9 days in vitro) autaptic (Tovar & Westbrook, 2002) and primary (Groc et al. 2006, 2007) cultures. The reduction of lateral receptor mobility in culture is due to an increase in extracellular matrix protein–receptor interaction and is proposed to be one of the hallmarks of synaptic maturation (Groc et al. 2009). In summary, although the mobility of NMDARs in immature neurons has been repeatedly shown, no evidence for NMDAR trafficking could be observed in our experiments in hippocampal slices from P35–42 rats.

The relationship between ambient glutamate, glutamate uptake and ES-NMDAR function has physiological and pathophysiological relevance for plasticity, learning and memory as well as the neurotoxicity underlying multiple neurological and psychiatric diseases (Kalivas, 2009; Lewerenz & Maher, 2015; Haroon et al. 2017; O’Donovan et al. 2017; Pinky et al. 2018). Our methodological approach overcomes some of the inherent difficulties of estimating relative proportions of synaptic and extrasynaptic NMDARs. It provides a new tool to assess ES-NMDAR function in physiological conditions as well as in slice models of various neuropsychiatric pathologies.

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### Additional information

#### Competing interests

The authors have no conflicting interests to declare.

#### Author contributions

C.P.B. and H.B. conceived the project. C.P.B., J.B. and A.M. designed the experiments. A.M. performed the experiments and analysed the data. A.M. and C.P.B. wrote the manuscript. J.B. and H.B. were involved in revising the intellectual content of the manuscript. The work was carried out at the Department of Neurobiology, University of Heidelberg, INF366, 69120 Heidelberg. All authors approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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acute brain slice, CA1 region, extracellular glutamate, extrasynaptic NMDA receptors, glutamate excitotoxicity, glutamate receptor antagonists, glutamate transporters, hippocampal pyramidal cells, N-methyl-D-aspartate, NMDARs, patch clamp, receptors

### Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

#### Statistical Summary Document

**Video S1:** Demonstration of the puff-suction technique.

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