Contribution of N-methyl D-aspartate receptors to synaptic function in rat hippocampal interneurons

Short title: NMDA receptors in interneurons

Authors:
Sam A Booker 1,2,3, Anna Sumera 1,2,3, Peter C Kind 1,2,3,4 and David J A Wyllie 1,2,3,4

Affiliations:
1Centre for Discovery Brain Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh, EH8 9XD, 2Simons Initiative for the Developing Brain, Hugh Robson Building, University of Edinburgh, Edinburgh EH8 9XD, UK, 3Patrick Wild Centre, Hugh Robson Building, University of Edinburgh, Edinburgh EH8 9XD, UK, 4Centre for Brain Development and Repair, Institute for Stem Cell Biology and Regenerative Medicine, Bangalore, 560065, India

Corresponding authors:
Sam A. Booker Centre for Discovery Brain Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh, EH8 9XD, UK, sam.a.booker@ed.ac.uk
David J. A. Wyllie Centre for Discovery Brain Sciences, Hugh Robson Building, University of Edinburgh, Edinburgh, EH8 9XD, UK, david.j.a.wyllie@ed.ac.uk

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Abstract

The ability of neurons to produce behaviourally relevant activity in the absence of pathology relies on the fine balance of synaptic inhibition to excitation. In the hippocampal CA1 microcircuit, this balance is maintained by a diverse population of inhibitory interneurons that receive largely similar glutamatergic afferents as their target pyramidal cells, with excitatory postsynaptic currents (EPSC) generated by both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs). In this study we take advantage of a recently generated GluN2A-null rat model to assess the contribution of GluN2A subunits to glutamatergic synaptic currents in three subclasses of interneuron found in the CA1 region of the hippocampus. For both parvalbumin (PV)-positive and somatostatin (SST)-positive interneurons, the GluN2A subunit is expressed at glutamatergic synapses and contributes to the EPSC. In contrast, in cholecystokinin (CCK)-positive interneurons, the contribution of GluN2A to the EPSC is negligible. Furthermore, synaptic potentiation at glutamatergic synapses on CCK-positive interneurons does not require the activation of GluN2A-containing NMDARs but does rely on the activation of NMDARs containing GluN2B and GluN2D subunits.

Significance Statement

NMDA receptors (NMDARs) are ionotropic glutamate receptors which play a critical role in interneuronal communication, and learning and memory. Despite much being known about NMDARs and the role different subunits play in controlling principal cell activity, less is known about their function in inhibitory interneurons. Here, we utilise a recently developed rat line where the key GluN2A receptor subunit is removed, combined with subunit-specific pharmacology to determine the synaptic properties and role of NMDAR subunits in interneuron function. Notably, we show that cholecystokinin (CCK) containing interneurons lack synaptic GluN2A and that long term potentiation at glutamatergic synapses on them is mediated by GluN2D subunits. Our findings have ramifications for the aetiology of neuropathological states and basic properties of brain function.

Introduction

Neuronal networks require finely balanced excitatory glutamatergic and inhibitory GABAergic synaptic transmission to allow learning, memory, and typical brain function. Alterations to this balance may result in autism and intellectual disability (Antoine et al., 2019), epilepsy (Sloviter,
1987), schizophrenia (Daskalakis et al., 2002), and depression (Czéh et al., 2015). This balance has been well-described in the CA1 of the hippocampus, where local inhibition arises from a heterogeneous population of inhibitory interneurons (INs) forming dense connections with themselves and excitatory pyramidal cells (PyrC; (Booker and Vida, 2018; Pelkey et al., 2017)).

INs possess diverse dendritic arbours, receiving glutamatergic inputs from both local and distant sources where synaptically released glutamate binds to and activates ligand-gated ion channels: chiefly α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs), that give rise to short (<10 ms) and long (100-1000 ms) synaptic events respectively (Cull-Candy et al., 2001). NMDARs have been proposed as the archetypal receptor underlying synaptic plasticity, and thus memory formation, in many cell types (Morris, 2013; Morris et al., 1990; Tsien et al., 1996).

NMDARs exist as tetrameric receptor complexes comprised of two GluN1 and two GluN2 subunits, the latter existing as four separate gene products (GluN2A-D) (Gielen et al., 2009; Wyllie et al., 2013). During early brain development, GluN2B and GluN2D are the dominant isoforms in the hippocampus (Flint et al., 1997; Martel et al., 2009; McKay et al., 2012; Monyer et al., 1994). In mature principal hippocampal neurons GluN2A and GluN2B subunits predominate while GluN2D subunits have been reported in some IN populations (Engelhardt et al., 2015; Garst-Orozco et al., 2020; Perszyk et al., 2016). Native NMDARs can exist as either diheteromers (i.e. GluN1/GluN2A) or as triheteromeric assemblies (i.e. GluN1/2A/2B) with ligand binding, receptor kinetics and ion channel conductance being conferred by the identity of the nature of the GluN2 subunits present (Gielen et al., 2009; Hansen et al., 2014; Stern et al., 1992; Wyllie et al., 1996). Indeed, the presence of GluN2A subunits confers fast kinetics, whereas the presence of GluN2B and GluN2D subunits confer increasingly slow kinetics ((Monyer et al., 1994; Vicini et al., 1998; Wyllie et al., 1998) and reviewed in (Wyllie et al., 2013)). Synaptically released glutamate, together with glycine (or D-serine), binds to synaptic and extrasynaptic NMDARs, and at depolarised membrane potentials the relief of voltage-dependent Mg$^{2+}$ block allows ion conduction (reviewed in Hansen et al. (2018)). This requirement of postsynaptic depolarisation and presynaptic neurotransmitter release for NMDAR opening defines them as a coincidence detector, critical for the establishment of Hebbian plasticity (Seeburg et al., 1995). Given these properties, the stoichiometry of NMDAR subunits confer distinct functional synaptic properties (Cull-Candy et al., 2001; Stern et al., 1992; Wyllie et al., 2013). Several studies have aimed to identify the contribution of NMDAR subunits to the different excitatory and inhibitory neuronal populations. The current
understanding is that mature CA1 PyrCs exclusively express GluN2A/B containing NMDARs (Flint et al., 1997; Gray et al., 2011). Meanwhile, hippocampal INs possess varied AMPAR- and NMDAR-mediated responses dependent on cell type (Akgül and McBain, 2016) and all major IN classes appear to express RNA for GluN2A/B/D subunits (Perszyk et al., 2016). Functional NMDARs have been shown on a variety of IN subtypes, including those expressing PV (Billingslea et al., 2014; Korotkova et al., 2010) and CCK (Kotzadimitriou et al., 2018), morphologically defined basket cells (BC) and dendritic inhibitory cells (Matta et al., 2013), oriens/alveus INs (Hajos et al., 2002), and neurogliaform neurons (Chittajallu et al., 2017). Despite this, many studies have been performed in juvenile rodent models and have not been able to determine the relative role of GluN2A in IN subtypes, due to the paucity of pharmacological modulators of this receptor subunit. Moreover, GluN2A-containing NMDARs are proposed to contribute to the induction of long-term potentiation (LTP) and GluN2B-containing NMDARs contribute to both long-term depression (LTD) and LTP (Liu et al., 2004; Volianskis et al., 2013), while GluN2D NMDARs may contribute to both short- and long-term plasticity (Tozzi et al., 2016; Volianskis et al., 2013). Thus, determining the functional properties of NMDARs in INs will provide insight into their synaptic recruitment and plasticity (Booker and Wyllie, 2021).

This study assesses the relative NMDAR mediated synaptic currents and the contribution of GluN2 subunits in morphologically and immunohistochemically identified hippocampal INs from wild-type and GluN2A null outbred rats. We achieve this by performing whole-cell patch-clamp recording from INs and PyrCs to examine the NMDAR-mediated synaptic current in the presence of pharmacological manipulation and the role of GluN2 subunits in the generation of synaptic plasticity.

**Methods**

**Animals**

All procedures were performed according to [Regional/Country] and [Local/Institute] guidelines and under the authority of [Licencing information]. GluN2A-null rats were generated through CRISPR/Cas9 deletion of the gene, with GluN2A protein loss shown previously (XXX, 2019). All rats were maintained on an outbred Long-Evans Hooded background, which were housed on a 12hr light/dark cycle with ad libitum access to food and water. Male rats were taken for recordings at 4-6 weeks of age, to avoid potential confounds due to onset of the oestrus cycle.
All recording were performed at 4-6 weeks of age. The average age of wild-type rats was 31.1 ± 0.5 days (N=38 rats total) and 32.4 ± 0.8 days (N=27 rats total) for GluN2A-null rats.

**Acute slice preparation**

Acute brain slices were prepared as previously described [XXX, 2017]. Rats were anesthetised with isofluorane, decapitated and their brains rapidly removed and placed in ice-cold carbogenated (95 % O\textsubscript{2}/5 % CO\textsubscript{2}) sucrose-modified artificial cerebrospinal fluid (sucrose-ACSF; in mM: 87 NaCl, 2.5 KCl, 25 NaHCO\textsubscript{3}, 1.25 Na\textsubscript{2}HPO\textsubscript{4}, 25 glucose, 75 sucrose, 7 MgCl\textsubscript{2}, 0.5 CaCl\textsubscript{2}). 400 μm horizontal slices containing the hippocampus were cut on an oscillating blade vibratome (VT1200S, Leica, Germany). Slices were placed in a submerged holding chamber in sucrose-ACSF for 30 min at 35°C, then stored at room temperature.

**Whole-cell patch-clamp recordings**

For electrophysiological recordings, slices were transferred to a submerged recording chamber perfused with pre-warmed carbogenated ACSF (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO\textsubscript{3}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 25 glucose, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}) at a flow rate of 4-6 mL.min\textsuperscript{-1} at 30±1°C). Slices were visualised under infrared differential inference contrast microscopy with a digital camera (Orca 2, Hamamatsu, Japan & SciCamPro, Scientifica, UK) mounted on an upright microscope (BX61-WI, Olympus, Japan & Slicescope, Scientifica, UK) with a 40x water-immersion objective lens (1.0 N.A., Olympus, Japan). Whole-cell patch-clamp recordings were performed with a Multiclamp 700B (Molecular Devices, CA, USA) amplifier. Recording pipettes were pulled from borosilicate glass capillaries (1.7 mm outer/1 mm inner diameter, Harvard Apparatus, UK) on a horizontal electrode puller (P-97, Sutter Instruments, CA, USA). For recordings, pipettes were filled with either a Cs-gluconate based (in mM: 140 Cs-gluconate, 4 CsCl, 0.2 EGTA, 10 HEPES, 2 MgATP, 2 Na\textsubscript{2}ATP, 0.3 Na\textsubscript{2}GTP, 10 Na\textsubscript{2}phosphocreatine, 2.7 Biocytin, and 5 QX-314; pH=7.4, 290-310 mOsm) or a K-gluconate based (in mM: 142 K-gluconate, 4 KCl, 0.5 EGTA, 10 HEPES, 2 MgCl\textsubscript{2}, 2 Na\textsubscript{2}ATP, 0.3 Na\textsubscript{2}GTP, 10 Na\textsubscript{2}phosphocreatine, 2.7 Biocytin; pH=7.4, 290-310 mOsm) internal solution, which gave 3-5 MΩ pipette tip resistances upon filling. Cells were rejected if: required more than -200 pA holding current to maintain -70 mV voltage-clamp, series resistance started >30 MΩ, or series resistance changed by more than 25% over the course of the recording. The average change in series resistance was 11.6% (mean\textsubscript{start} = 18.7 ± 5.8 [range: 7.0 – 29.9]; mean\textsubscript{end} = 18.4 ± 6.5 [range: 7.6 – 36.5]; p=0.45, paired Student’s t-test).
Recordings of pharmacologically isolated excitatory postsynaptic current (EPSC) were performed whole-cell following wash-in of Cs-gluconate solution (~2-5 minutes) to provide optimal voltage-clamp, in the presence of picrotoxin (50 µM). EPSCs were generated by a twisted Ni:Chrome bipolar wire placed in either str. radiatum (CA1 PyrC, PV-INs, CCK-INs) or in the alveus (SSt INs), reflecting major synaptic inputs to different cell types (0.1 ms stimulus duration, 20.3 ± 18.3 V stimulus). Stimuli were delivered via constant voltage stimulator (Digitimer, Cambridge, UK) sufficient to produce a monosynaptic EPSC in the range of ~200 pA. The amplitude of these monosynaptic AMPA-EPSCs varied between cell-types (p=0.0016, 1-way ANOVA) but not within cell type (CA1 PCs: p=0.84; PV IN: p=0.83; p=0.84; p=0.69; Holm-Sidak tests). For AMPAR-EPSCs 10 traces were collected at 20 second intervals at -70 mV. For NMDAR-EPSCs, either CNQX was bath applied first and AMPAR-EPSC blockade observed, or the cell was held at +40 mV to identify the mixed AMPAR/NMDAR-EPSC, and then CNQX applied. All NMDAR-EPSCs were recorded following full wash-in of CNQX at +40 mV using the same stimulation intensity as for AMPAR-EPSCs. Pharmacology for the specific NMDAR subunits: GluN2B – ifenprodil tartrate (10 µM) or NAB-14 (10 µM) were bath-applied. As NAB-14 is highly lipophilic (Yi et al., 2019) all tubing was rinsed with 100% ethanol and liberal amounts of distilled water between recordings to ensure full removal of the drug from the surface of perfusion tubes. All EPSC amplitudes were recorded at the peak of the EPSC, as measured over a 2 ms peak average. All rise-times are reported as the 20-80% of the peak EPSC amplitude. Decay time-constants were calculated from a mono-exponential (AMPAR-EPSC) or bi-exponential (NMDAR-EPSC) curve fit to the decaying phase of the EPSC. For bi-exponential fits, the weighted tau was taken as the decay time-constant. For all kinetic properties, only NMDAR EPSCs with amplitude greater than 20 pA were included for analysis, to exclude measurement artefacts. NMDAR/AMPA EPSC amplitude ratios were calculated from the average EPSC recorded for the respective epoch.

For LTP recordings, cells were recorded in current-clamp, using a K-gluconate based internal solution. Putative CCK INs were selected for recording in str. radiatum and monosynaptic excitatory postsynaptic potentials (EPSPs) were generated via bipolar electrode stimulation of the str. radiatum, placed approximately 500 µm distal to the recorded somata. Following breakthrough into whole-cell configuration, the membrane potential was set to -70 mV with the application of a bias current, and the bridge balanced. Then EPSPs with amplitude of approximately 5 mV (Mean: 5.94 ± 2.85 mV, Range: 1.64 – 12.35 mV) were recorded for 5 minutes of stable baseline (<10% change over 5 minutes, calculated from linear fit of baseline.
period), if stability was not achieved within 7.5 minutes of breakthrough, the recording was abandoned. Following 5 minutes of stable recording, LTP was induced, with 2x 100 Hz tetanus of stimuli, at the same strength as the baseline EPSP. Following induction, 25 minutes of EPSPs were recorded to assess the potentiation of the whole-cell EPSP, reported as the peak amplitude measured as the peak 2 ms response. All recordings were filtered online at 10 kHz with the built-in 4-pole Bessel Filter and digitized at 20 kHz (Digidata1440, Molecular Devices, CA, USA). Traces were recorded in pCLAMP 9 (Molecular Devices, CA, USA) and stored on a personal computer. Analysis of electrophysiological data was performed offline using the open source software package Stimfit (Guzman et al., 2014).

Following recording, all cells were resealed by carefully withdrawing the patch-pipette in a manner akin to forming an outside-out patch. The slices were then fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH=7.35) overnight at 4°C. Slices were then transferred to 0.1 M phosphate buffered saline (PBS) until processed for immunohistochemistry.

Histological processing and imaging:
Immunolabelling of recorded neurons was performed as previously described [XXX, 2014], slices were washed several times in PBS, then blocked in 10% normal goat serum (NGS), 0.3% TritonX-100, 0.05% NaN$_3$ in PBS, for 1 hour at room temperature. Slices were then incubated with primary antibodies for either PV (monoclonal mouse, 1:5000, SWANT, Bellizona, Switzerland), pre-pro-CCK (polyclonal rabbit, 1:1000, Frontiers Laboratory, Japan), or SSt (polyclonal rabbit, 1:2000, Peninsula Laboratories, USA). Primary antibodies were diluted in PBS containing 5% NGS, 0.3% TritonX-100 and 0.05% NaN$_3$, for 72 hours at 4°C. Slices were thoroughly washed with PBS and then secondary antibodies (anti-mouse or anti-rabbit; 1:1000 AlexaFluor488, Invitrogen, UK) were applied with streptavidin conjugated to AlexaFluor633 (1:1000, Invitrogen, UK) applied diluted in PBS containing 3% NGS, 0.1% TritonX-100 and 0.05% NaN$_3$, for 3 hrs at room temperature or 24 hrs at 4°C. Slices were rinsed in PBS, then PB and mounted on glass slides with Vectashield hard-set mounting medium (H1400, Vector Labs, UK). For CA1 PyrCs, the same protocol was used, albeit with Streptavidin AlexaFluor633 applied at 1:1000.

Confocal image stacks were collected on an Axiovert LSM 510 (Zeiss, Germany) invert scanning-confocal microscope equipped with a 20x (N.A. 0.45, Zeiss, Germany) air-immersion
or 63x (N.A. 1.4, Zeiss, Germany) oil-immersion objective lenses. For identification of neurons Z-stacks (1 µm steps, 1024x1024 pixels) containing the somatodendritic axis, and axon distribution were taken. For neurochemical identification, the somata was imaged under high-magnification and immunolabelling assessed over the somatic focal plane. All image analysis was performed with the FIJI package of ImageJ. For reconstruction, the neurons were imaged fully then stitched, segmented, and rendered offline in FIJI, using the Simple Neurite Plugin (Longair et al., 2011).

Statistical analysis
All experiments and analysis were performed blind to genotype. Throughout this study data are shown as mean ± standard deviation (SD) and number of cells (n) and animals (N) indicated. The required sample size was estimated based on an assumed effect size of 20% with 15% SD at 80% power, giving a required N as 7-10 per group. Some data showed >20% effect size, thus requiring fewer biological replicates (N). All data are reported as animal averages, unless stated otherwise, and shown alongside estimation statistics of difference between means and confidence intervals in the form (r-value, effect size; 95% confidence interval, p-value, test-performed), the usefulness of which has been demonstrated previously (Manouze et al., 2019). We performed statistical comparisons of effect size in a paired or unpaired manner, using either Student’s t, Mann-Whitney, or Wilcoxon signed-rank tests, depending on whether data were normally distributed; which was confirmed with the Anderson-Darling test. For group analysis, 1-way ANOVA was performed. Statistical significance was assumed if p<0.05.

Results
Absence of GluN2A subunits leads to slowed kinetics of NMDAR-EPSCs in CA1 PyrCs
To first confirm that loss of GluN2A receptors leads to functional changes in NMDAR signalling we made recordings from identified CA1 pyramidal cells (PyrC) (Figure 1A) in both wild-type (n=20 cells; N=13 rats) and GluN2A-null littermate rats (n=21 cells; N=11 rats). In all recordings, large AMPAR mediated EPSCs were recorded in response to electrical stimulation of putative Schaffer-collateral afferents in str. radiatum (Figure 1B). These EPSCs had an average amplitude of 357 ± 198 pA in wild-type rats, similar to that of 509 ± 228 pA in GluN2A-null rats (r = 0.12; -152; CI: -332, +29, p=0.095, unpaired Student’s t-test). AMPAR rise-time was 2.1 ± 0.7 ms and had a decay time-constant of 8.5 ± 1.7 ms in wild-type rats, which was very similar in the GluN2A-null PyrCs (rise-time: r = 0.002; +0.053; CI: -0.46, 0.57; p=0.83; decay...
time-constant: $r = 0.03; +0.80; CI: -1.23, 2.82; p=0.42$, unpaired Student’s t-tests). When recorded at +40 mV in the presence of 10 µM CNQX (10 µM), we consistently observed NMDAR-EPSCs (Figure 1B), which in wild-type rats had a rise-time of 3.6 ± 0.7 ms and a weighted decay time-constant of 120.5 ± 39.4 ms. The average NMDAR-EPSC had an amplitude of 129 ± 73 pA, giving rise to an average NMDAR/AMPAR ratio of 0.43 ± 0.22. Consistent with GluN2A subunits conferring rapid gating kinetics to NMDARs, we observed a slowing of NMDAR-EPSCs in the GluN2A-null rat (Figure 1B) with 20-80% rise-time slowed by 27% ($r = 0.15; +0.55; CI: -0.03, 1.12; p=0.06$, unpaired Student’s t-test; Figure 1C) and the decay time-constant slowed by 75% ($r = 0.13; +66.1; CI: 32.3, 99.9; p=0.0005$, unpaired Student’s t-test; Figure 1D). We observed a 28% reduction in the NMDAR/AMPAR ratio associated with loss of the GluN2A subunit, which was not significantly different ($r = 0.43; -0.13; CI: -0.28, 0.02; p=0.08$, unpaired Student’s t-test; Figure 1E), suggesting that the total number of NMDARs may be reduced at Schaffer-collateral synapses. Together, these data confirm that NMDARs in CA1 PyrCs contain GluN2A subunits, which contribute to the kinetics properties of the receptor.

**NMDARs in hippocampal INs express variable levels of GluN2A**

We next assessed the contribution of GluN2A subunits to synaptic NMDARs in identified hippocampal INs. We first sought to identify EPSCs in PV INs, which were located in and around str. pyramidale, with large somata with multipolar dendrites. These were initially selected based on low membrane resistance and fast membrane decay times upon breakthrough into the whole-cell configuration. For the current analysis we identified 20 PV INs from 15 wild-type rats, and 14 PV INs from 13 GluN2A-null rats, which all displayed clear immunolabelling for PV. In wild-type rats, 50% (n=10 cells) of PV INs were identified BCs with axons localised to str. pyramidale (Figure 2A) and 35% (7 cells) had axon localised to str. radiatum and oriens, thus representing likely bistratified cells, the remaining 3 cells had axons cut close to the soma thus preventing further identification. In GluN2A-null rats, 79% of recovered PV INs were BCs (n=11 cells), 14% were bistratified (n=2 cells), and one cell could not be identified due to a proximally cut axon.

In PV INs, stimulation of str. radiatum in the presence of picrotoxin (50 µM) at -70 mV gave rise to AMPAR-mediated EPSCs with amplitudes of 373 ± 306 pA in wild-type and 350 ± 166 pA in GluN2A-null rats ($r = 0.003; 24.1; CI: -173, 221; p=0.80$, unpaired Student’s t-test), with similar rise-time (WT: 1.6 ± 1.6 ms, GluN2A-null: 1.6 ± 1.3 ms, $r = 0.0007; -0.08; CI: -0.08, 0.56$;
$p=0.80$, unpaired Student’s t-test) but 33% longer decay time-constants (WT: $6.7 \pm 2.1$ ms, GluN2A-null: $9.0 \pm 3.4$ ms, $r=0.15$; 2.3; CI: 0.06, 4.5; $p=0.05$, unpaired Student’s t-test). NMDAR-mediated EPSCs, recorded at $+40$ mV in PV INs from WT rats (Figure 2B) had an amplitude of $76 \pm 62$ pA, 20-80% rise-time of $3.2 \pm 0.7$ ms (Figure 2C), and decay time-constant of $90.1 \pm 28.4$ ms (Figure 2D), which was ~30 ms faster than those of CA1 PyrCs ($r=0.17$; 30.4; CI: -60.0, -0.76; $p=0.045$, unpaired Student’s t-test). Due to thresholding the NMDAR-EPSC at 20 pA for kinetic measurements 3 animals were excluded from these analyses. The NMDAR/AMPAR ratio for wild-type PV INs was $0.26 \pm 0.17$, lower than that of CA1 PyrCs ($r=0.16$; -0.17; CI: -0.32, -0.01; $p=0.038$, unpaired Student’s t-test). In terms of cell-type specific effects, the decay time-constants of PV BCs (109.2 ± 21.1 ms) tended to be longer than that of PV bistratified cells ($78.0 \pm 36.1$ ms; $r=0.25$; -31.3; CI: -69.3, 6.8; $p=0.097$, unpaired Student’s t-test), despite similar NMDAR/AMPAR ratios ($r=0.007$; 0.035; CI: -0.21, 0.28; $p=0.77$, unpaired Student’s t-test). All kinetic data for identified subtypes are shown in Table 1.

Compared to wild-type, NMDAR-EPSCs recorded in PV INs from GluN2A-null rats had similar amplitudes of $51 \pm 46$ pA ($r=0.54$; -25.1; CI: -68.5, 18.3; $p=0.25$, unpaired Student’s t-test) and rise-times of $3.1 \pm 1.2$ ms ($r=0.001$; -0.06; CI: -0.91 - 0.79; $p=0.89$, unpaired Student’s t-test). Consistent with the contribution of GluN2A subunits to NMDAR-EPSCs evoked in PV INs, we measured an 75% increase in the decay time-constant to $158 \pm 50$ ms in GluN2A-null rat compared to wild-type ($r=0.42$; 67.5; CI: 32.2, 102.8; $p=0.0007$, unpaired Student’s t-test; Figure 2D). PV INs in GluN2A-null rats had NMDAR/AMPAR ratios of $0.17 \pm 0.14$, which was lower but not significantly different from WT ($r=0.084$; -0.09; CI: -0.21, 0.03; $p=0.14$, unpaired Student’s t-test, Figure 2E). Although not statistically different between genotypes, the decay kinetics for identified BCs and bistratified cells generally obeyed the population average (Table 1). Consistent with their decay time-constants in wild-type rats, BCs showed a ~50% slowing of the NMDAR-EPSC decay in GluN2A-null rats, while bistratified cells showed 200% slowing in decay time-constants (Table 1). These data suggest that GluN2A containing NMDARs contribute to synaptic currents in PV-INs, comparable to that of CA1 PyrCs, with noted differences between morphological subtypes identified.

We next asked if the canonical feedback INs expressing SSt (Figure 3A) (Booker et al., 2018), possessed NMDAR-EPSCs mediated by GluN2A; all cells included in our displayed clear immunolabelling for SSt at the level of the soma (Figure 3A, inset). The major synaptic inputs
to SST INs arise from local CA1 PyrCs (Blasco-Ibanez and Freund, 1995), as such we used an 
*alveus* extracellular stimulation to elicit monosynaptic AMPAR- and NMDAR-EPSCs (Figure 
3B), as *str. radiatum* stimulation would lead to disynaptic responses. AMPAR-EPSCs in SST INs 
had comparable amplitudes of 256 ± 178 pA in wild-type (n= 17 cells, N=14 rats) and 
194 ± 117 pA in GluN2A-null rats (n=17 cells, N=12 rats, r = 0.043; 62.4; CI: -62.2, 187.0; 
p=0.31, unpaired Student’s t-test). Neither 20-80% rise-time (r = 0.074; 0.51; CI: -0.27, 1.28; 
p=0.38, Mann-Whitney test) nor decay time-constant (r = 0.09; 3.43; CI: -1.17, 8.02; p=0.14, 
unpaired Student’s t-test) of AMPAR-EPSCs were different between genotypes.

<table>
<thead>
<tr>
<th>Cell type</th>
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<td>BC</td>
<td>14</td>
<td>7</td>
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<tr>
<td>SST</td>
<td>SCA/ADA</td>
<td>15</td>
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<td>SST</td>
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**TABLE 1: Key properties of NMDAR-mediated EPSCs in PyrC and IN morphotypes in CA1**

**of the hippocampus.** Summary of NMDAR mediated EPSC decay time-constant and 
NMDAR/AMPAR ratio from identified morphotypes of hippocampal INs. Statistics shown as p- 
values reported from unpaired Student’s two-tailed t-tests, where statistical significance was 
observed this has been indicated (bold and underlined). Data are shown as mean ± SD.

In wild-type rats, NMDAR-EPSCs had an average amplitude of 80 ± 69 pA. NMDAR-EPSCs 
had 20-80% rise-time of 2.8 ± 0.8 ms (Figure 3C), decay time-constants of 85 ± 28 ms (Figure 
3D), and a NMDAR/AMPAR ratio of 0.43 ± 0.45 (Figure 3E). In 7 SST INs it was not possible to
measure an NMDAR-EPSC kinetics, due to their failure to reach the threshold 20pA amplitude for such measurements. The measured decay time-constants of SST INs were 30% faster than for CA1 PyrCs (r = 0.21; -35.3; CI: -67.2, -3.4; p=0.032, unpaired Student’s t-test). In GluN2A-null rats, we observed similar amplitude NMDAR-EPSCs (68 ± 54 pA, r = 0.009; -0.01; CI: -0.38, 0.35; p=0.94, unpaired Student’s t-test), which resulted in a comparable NMDAR/AMPAR ratio of 0.41 ± 0.44 (r = 0.0002; -35.3; CI: -67.2, -3.4; p=0.90, Mann-Whitney test Figure 3C). These NMDAR-EPSCs had similar rise-times of 3.0 ± 0.8 (r = 0.015; 0.19; CI: -0.65, 1.04; p=0.63, unpaired Student’s t-test, Figure 3C), but had decay time-constants of 159 ± 60 ms, 86% longer than wild-type (r = 0.41; 73.5; CI: 26.7, 120.2; p=0.004, unpaired Student’s t-test; Figure 3D).

These properties were consistent when tested within the only morphotype identified in this study, the so-called Oriens/Lacunosum-Molecular (OLM) cell, which had decay kinetics overlapping with the population average of all SST INs (Table 1). Together, these data suggest that NMDAR-EPSCs are mediated by GluN2A subunits to a large extent in SST INs.

A major subtype of hippocampal IN are those expressing the neuropeptide CCK (Booker and Vida, 2018), which likely reflect caudal ganglionic eminence (CGE) INs previously identified to contain high levels of NMDARs (Matta et al., 2013) and proposed to express GluN2A mRNA (Persyk et al., 2017). To determine the contribution of GluN2A to synaptic currents in these neurons, we performed recordings of AMPAR- and NMDAR-EPSCs from identified CCK INs in wild-type (n=56 cells, N= 27 rats) and GluN2A-null (n=23 cells, N= 16 rats), which were all confirmed with pre-pro-CCK immunolabelling (Booker et al., 2017). The cells recovered comprised BCs (n=15, Figure 4A), Schaffer-collateral-associated/apical dendrite-associated cell (SCA/ADA, n=21), and performant path-associated (PPA, n=6 cells) cells in wild-type rats, the remaining cells (n=14) had their axon close to the cell body, preventing further classification.

In GluN2A-null rats we positively identified BCs (n=7), SCA/ADA cells (n=7), PPA cells (n=2) the remaining cells had a cut axon (n=7), thus could not be classified.

AMPAR-EPSCs recorded from CCK INs in wild-type rats had an average amplitude of 204 ± 90 pA, which were similar to that recorded in GluN2A-null rats (262 ± 129, r = 0.06; -57.1; CI: -124.8 to 10.56; p=0.10, unpaired Student’s t-test). There were no observed differences in AMPAR-EPSC 20-80% rise-time (r = 2 x 10^5; 0.02; CI: -1.68, 1.73; p=0.66, Mann-Whitney test) or decay time-constant (r = 4 x 10^-4; -0.29; CI: -4.59, 4.02; p=0.86, Mann-Whitney test).

NMDAR-EPSCs were elicited as for PyrCs and PV INs, by stimulation of str. radiatum, in the presence of picrotoxin at +40 mV (Figure 4B). In wild-type rats, the average NMDAR-EPSC had
an amplitude of 99 ± 48 pA, with rise-time of 3.9 ± 1.4 ms (Figure 4C), decay time-constant of
170 ± 64 ms (Figure 4D) and a NMDAR/AMPA ratio of 0.63 ± 0.36 (Figure 4E). There was no
apparent difference in NMDAR-EPSC properties observed between morphotypes of CCK INs
(Table 1). Comparison of NMDAR-EPSCs in the GluN2A-null rats (Figure 4B) did not indicate a
change in rise-time (3.7 ± 1.0 ms; r = 0.01; -0.27; CI: -1.11, 0.57; p=0.46, Mann-Whitney test;
Figure 4C), increase in decay time-constant (152 ± 44 ms; r = 0.02; -17.5; CI: -54.4, 19.3;
p=0.27, Mann-Whitney test; Figure 4D), or NMDAR/AMPAR ratio (0.55 ± 0.36; r = 0.01; -0.07;
CI: -0.30, 0.16; p=0.52, unpaired Student’s t-test; Figure 4E). Compared to CA1 PyrCs, the
NMDAR EPSC decay time-constant was 41% longer in CCK INs (r = 0.15; -49.2; CI: -88.5 to -
9.8; p=0.012, Mann-Whitney test), and the NMDAR/AMPAR ratio tended to be larger, albeit not
significantly so (r = 0.08; -0.20; CI: -0.42, 0.02; p=0.052, Mann-Whitney test). Together, these
data suggest that CCK INs possess high level of NMDARs with markedly different kinetics from
CA1 PyrCs and appear to lack GluN2A subunits.

Differential ifenprodil sensitivity of NMDARs indicates divergent GluN2B expression
The data presented so far indicate that in CA1 PyrCs, PV and SSt INs GluN2A subunits
contribute to synaptic NMDAR-EPSCs, but that effects of GluN2A loss are largely absent at
functional synaptic receptors on CCK INs. We next sought to determine the relative expression
of GluN2B subunits, through their pharmacological blockade with the selective GluN2B
antagonist ifenprodil (10 µM; (Lei and McBain, 2002); Williams (2001)). In all cell-types tested,
we observed a degree of ifenprodil sensitivity, when applied to pharmacologically isolated
NMDAR-EPSCs (Figure 5A). Blockade of NMDAR-EPSCs mediated by ifenprodil was use-
dependent, as previously described (Kew et al., 1996), and developed slowly in all cells tested
(Figure 5B). In CA1 PyrCs, following 10 minute wash-in, ifenprodil produced a strong 55 ± 21%
block of native NMDAR-EPSCs reducing the average amplitude from 136 ± 78 to 54 ± 30 pA
(N=13 rats; r = 0.60; -82.4; CI: -137.4, -27.4; p=0.009, paired Student’s t-test; Figure 5C),
consistent with the presence of GluN2B receptor subunits (Gray et al., 2011, Li et al., 2019) but
a greater block than for triheteromeric receptors alone (Hansen et al., 2014). This ifenprodil
block in CA1 PCs was stronger in the GluN2A-null rat at 83 ± 13% (N=8 rats; r = 0.48; -25.2; CI:
-48.7, -1.7; p=0.039, paired Student’s t-test), consistent with a loss of GluN2A subunit
containing triheteromeric receptors (Hansen et al., 2014). In PV INs, the average NMDA EPSC
amplitude was blocked by 55 ± 24%, from 88 ± 65 pA to 39 ± 33 pA (N=12 rats; r = 0.61; -49.5;
CI: -79.4, -19.6; p=0.005, paired Student’s t-test; Figure 5C). PV INs in the GluN2A-null rat
(N=3 rats) displayed a 70 ± 12% ifenprodil block, which was statistically not different from wild-
type ($r = 0.09; 15.0; CI: -17.7, 47.6; p=0.34$, unpaired Student’s t-test). In contrast, SSt INs displayed a $34 \pm 13\%$ block, with NMDAR-EPSCs reduced from $145 \pm 46 \text{pA}$ to $107 \pm 42 \text{pA}$ (N=5 rats; $r = 0.55; -37.3; CI: -83.7, 9.1; p=0.094$, paired Student’s t-test), which increased to a $57 \pm 14\%$ block in the GluN2A-null rats (N=6, $r = 0.47; 23.1; CI: 5.9, 40.3; p=0.014$, unpaired Student’s t-test). CCK INs displayed a $42 \pm 23\%$ block from $126 \pm 60 \text{pA}$ to $64 \pm 38 \text{pA}$ (N=12 rats, $r = 0.52; -30.6; CI: -47.4, -13.8; p=0.002$, paired Student’s t-test). In GluN2A-null rats (N=3), ifenprodil produced a block of $57 \pm 10\%$, which was not different to that of wild-type ($r = 0.07; 15.2; CI: -13.4, 43.8; p=0.28$, unpaired Student’s t-test). Comparison of the ifenprodil block between cell types revealed there was no substantial difference between cell types in wild-type rats (F= 1.864, p=0.15, One-way ANOVA, Figure 5C). In all but CCK INs, the degree of ifenprodil block tended to be greater in the GluN2A-null rat, indicating a greater contribution of this subunit to synaptic NMDARs (Figure 5D). These data suggest that GluN2B sensitivity is broadly equivalent between PyrCs, PV, SSt, and CCK INs, and that GluN2B subunits contribute to heterotrimeric NMDARs in all cell classes.

**NMDARs in CCK INs are GluN2D-containing and contribute to LTP induction**

From the data we have shown so far, CCK INs NMDAR-EPSCs appears to be unaffected by the loss of GluN2A. Furthermore, CCK INs express high levels of GluN2D mRNA (Perszyk et al., 2016) and *str. radiatum* INs also possess NMDAR-EPSCs sensitive to GluN2C/D modulation (Perszyk et al., 2016; Swanger et al., 2018; Yi et al., 2019). To determine if GluN2D-containing receptors specifically contribute to NMDAR-EPSCs in CCK INs, we utilised the GluN2C/D negative allosteric modulator NAB-14 (Swanger et al., 2018). As GluN2C is expressed only minimally in hippocampal neurons (Perszyk et al., 2016; Wenzel et al., 1997), it likely that the effects of NAB-14 can be attributed to NMDAR containing GluN2D subunits. Bath application of NAB-14 (10 µM) to recordings of pharmacologically isolated NMDAR-EPSCs substantially reduced their amplitude in CCK INs from wild-type and GluN2A-null rats (Figure 6A). Following 10 minutes wash-in of NAB-14, NMDAR-EPSCs were reduced by 65%, (n=13 cells, $r = 0.53; -75.2; CI: -119.9, 30.5; p=0.0002$, Wilcoxon test), and reduced by 37% in GluN2A null rats (n=5 cells, $r = 0.81; -20.4; CI: -33.9, -6.8; p=0.014$, paired Student’s t-test), which was not statistically different between genotypes ($r = 0.19; -23.7; CI: -50.0, 2.6; p=0.074$, unpaired Student’s t-test). Bath application of NAB-14 did not alter the 20-80% rise-time of NMDAR-EPSCs in either wild-type ($r = 0.007; 0.30; CI: -2.1, 2.7; p=0.79$, paired Student’s t-test) or GluN2A-null CCK INS ($r = 0.097; -1.18; CI: -0.92, 0.57; p=0.55$, paired Student’s t-test). As expected from a selective block of GluN2D containing receptors, the decay time-constants of NMDAR-EPSCs were...
shortened by 42% for wild-type \( r = 0.61; -109.7; CI: -168.3, -51.0; p=0.002, \) paired Student’s t-test), and by 50% for CCK INs in GluN2A-null rats \( r = 0.67; -98.5; CI: -194.3, -2.7; p=0.046, \) paired Student’s t-test); the magnitude of this effect was not different between genotypes \( r = 0.006; -3.8; CI: -30.3, 22.7; p=0.046, \) paired Student’s t-test). Taken together, these data reveal that NMDAR-EPSCs in CCK INs result from activation of NMDARs composed of GluN2D and GluN2B, but not GluN2A subunits.

NMDARs are known to mediate Hebbian LTP, in both PyrCs (Malenka, 1991) and INs (Lamsa et al., 2005; Lamsa et al., 2007). Indeed, GluN2A/B containing receptors have been shown to induce LTP (Berberich et al., 2005), with the GluN2A subunit presumed requisite for LTP induction (Liu et al., 2004). Given the absence of GluN2A from CCK INs, we asked if the presence of GluN2D containing NMDARs was required to induce the Hebbian form of LTP present in these INs (Lamsa et al., 2007). In whole-cell recordings from CCK INs, performed in current-clamp, monosynaptic excitatory postsynaptic potentials (EPSPs) were evoked from putative Schaffer-Collateral afferents in *str. radiatum*, in the presence of picrotoxin (50 µM). The average amplitude of these EPSPs was 5.4 ± 1.9 mV in wild-type CCK INs (N=9 rats). Following 5 minutes of baseline recording, LTP was induced by delivering 2x 100 Hz trains of stimuli (1 second duration) to the stimulated pathway, the EPSP amplitude was then measured for 25 minutes after induction (Figure 6C). In control recordings from wild-type CCK INs, this protocol resulted in potentiation of the EPSP to 59 ± 30% increase from control EPSP at 25 minutes \( r = 0.76; 3.4; CI: 1.9, 5.0; p=0.001, \) paired Student’s t-test, Figure 6D, 6E). In recordings from wild-type rats (N=6 rats), NAB-14 (10 µM) was pre-applied (10 minutes before recording), then CCK INs recorded. The baseline EPSPs in the presence of NAB-14 had an average amplitude of 6.5 ± 3.4 mV, not different from control EPSPs \( r = 0.05; 1.1; CI: -1.9, 4.1; p=0.45, \) unpaired Student’s t-test). Following the same induction as for control recordings, in the presence of NAB-14, little to no potentiation of the EPSP amplitude (6 ± 37% increase) was observed at 25 minutes after tetanization \( r = 0.004; 0.14; CI: -2.2, 2.5; p=0.89, \) paired Student’s t-test, Figure 6D, 6E), which was markedly lower than the potentiation observed in control recordings \( r = 0.46; -57.7; CI: -95.0, -20.3; p=0.005, \) unpaired Student’s t-test). To confirm that LTP in CCK INs was independent of GluN2A, we performed the same recordings in GluN2A-null rats. Following the same induction paradigm, we observed sustained synaptic potentiation in CCK INs from the GluN2A-null rat, with a 134 ± 98% increase in EPSP amplitude under control conditions (N= 8 rats, \( r = 0.53; 7.4; CI: 1.1, 13.6; p=0.008, \) Wilcoxon test, Figure 6F, 6G).

Contrary to LTP being reduced in the absence of GluN2A, the magnitude of EPSP potentiation...
was 48% greater in CCK INs of the GluN2A-null rat ($r = 0.25; 76.0; \text{ CI: } 3.1, 149.0; p=0.042$, unpaired Student’s t-test). In the presence of NAB-14, there was no potentiation of the EPSP when measured at 25 minutes in CCK INs from the GluN2A-null rats (N= 6 rats, -36 ± 46%, $r = 0.092; -0.56; \text{ CI: } -2.6, 1.5; p=0.51$, paired Student’s t-test, Figure 6F, 6G). These data reveal that LTP at Schaffer-collateral synapses onto CCK INs in part depend on GluN2D but not GluN2A-containing NMDARs.

Discussion

In the current study we provide evidence for GluN2A-specific modulation of synaptic NMDAR-mediated currents in hippocampal INs and PyrCs. We show that NMDAR-EPSCs in PV INs closely resemble that of CA1 PyrCs, both in terms of kinetics, synaptic contribution, and GluN2A/2B composition. The closely related cell type, SST INs display NMDAR-EPSCs that are typically faster and likely composed largely of GluN1/2A or GluN1/2A/2B receptors. Finally, we show unequivocally that CCK INs lack a prominent GluN2A component to their NMDAR-EPSCs; rather their synaptic NMDARs are likely composed of GluN1/2B/2D triheteromeric receptors based on ifenprodil and NAB-14 sensitivity. Finally, we confirm that these NMDARs in CCK INs are required to induce a Hebbian form of LTP. Together, the data we present provide further evidence that different IN subtypes display divergent synaptic NMDAR pharmacology.

Functional identification of GluN2 subunits in hippocampal neurons:

CA1 PyrC have been postulated to express both GluN2A/B triheteromeric receptors and GluN2A or GluN2B diheteromeric receptors in mature neurons (Al-Hallaq et al., 2007; Gray et al., 2011; Tovar et al., 2013; Traynelis et al., 2010). In this study we provide evidence that CA1 PyrCs likely express at least both GluN2B diheteromeric and GluN2A/B triheteromeric receptors at functional synapses in late juvenile rats. NMDAR-mediated EPSCs were significantly slowed in both rise and decay phase following loss of GluN2A subunits, but did not have altered amplitudes. The observed 50-60% block produced by Ifenprodil in wild-type PyrCs is consistent with the pharmacology of GluN1/2A/2B triheteromeric NMDARs observed both heterologous cell lines (Erreger et al., 2005; Hatton and Paoletti, 2005; Marwick et al., 2019) and also cultured dissociated neurons (Martel et al., 2009; McKay et al., 2012). Indeed, the increased block observed in the GluN2A-null rat, is consistent with a switch to GluN1/2B diheteromeric receptors at synapses (Gray et al., 2011; McKay et al., 2012). Nevertheless, ifenprodil has been suggested to block GluN2A-containing receptors to some degree in cultured neurons (Kew et
however the IC50 at NMDARs only comprising the Glu2A receptor is an order of magnitude higher than have we used in this study (Avenet et al., 1996). Indeed, the ifenprodil block we observe is greater in the absence of GluN2A, consistent with a selective effect at GluN2B-containing NMDARs. Furthermore, a range of ifenprodil concentrations up to and including 10 µM have been employed in ex vivo slice preparations, with overall findings consistent with GluN2B selective effects (Lei and McBain, 2002; Matta et al., 2013). Taken together these data show in late juvenile rats that synaptic NMDA receptors are likely composed of a combination of GluN2A/B triheteromeric or GluN2B diheteromeric receptors, which accounts for the slower kinetics and tendency towards reduced NMDAR/AMPAR ratios at Schaffer-collateral synapses in these neurons.

The role of NMDARs in the signalling, plasticity, and excitability of hippocampal INs is poorly understood, and even less so the contribution of different NMDAR subunits to these functional properties (Akgül and McBain, 2016; Booker and Wyllie, 2021; Moreau and Kullmann, 2013). Many studies have suggested that PV INs likely possess NMDAR-mediated EPSCs with about 4-fold lower amplitudes compared to AMPAR-mediated EPSCs (Koh et al., 1995; Korotkova et al., 2010; Matta et al., 2013). In the neocortex such NMDAR-EPSCs possess a GluN2A component in the neocortex (Picard et al., 2019), and display a low GluN2B content in the hippocampus of juvenile mice (Matta et al., 2013). We show that CA1 PV INs possess NMDAR of similar composition to CA1 PyrCs based on recordings in the GluN2A-null rat and in the presence of Ifenprodil. These NMDAR-EPSCs have faster kinetic properties than neighbouring CA1 PyrCs (Gray et al. (2011) and current data), which in the absence of reduced synaptic amplitudes in the GluN2A-null rat may relate to the altered dendritic properties of PV INs compared to CA1 PyrCs (Nörenberg et al., 2010) or altered NMDA effects on such (Camiré and Topolnik, 2014). Our finding that the amplitude of NMDARs-EPSCs in PV INs is comparable to CA1 PyrCs, with similar effect of GluN2A loss and GluN2B blockade, offer an explanation for the presence of readily inducible Hebbian or anti-Hebbian LTP in PV and SST INs class, reliant on the presence of NMDARs or calcium-permeable AMPARs respectively (Billingslea et al., 2014; Kullmann and Lamsa, 2007; Laezza et al., 1999; Lamsa et al., 2005; Perez et al., 2001; Szabo et al., 2012). Indeed, activating these NMDARs likely recruits similar signalling cascades to PyrCs (Berberich et al., 2005; Gray et al., 2011) with a defined role in plasticity induction (Camiré and Topolnik, 2014). Our data suggest that, in rats, NMDAR-EPSCs may be mediated by a greater proportion of GluN2B containing receptors than in mice, given the differential ifenprodil sensitivity observed (Matta et al., 2013), although this has some potential caveats.
The presence of large NMDAR-EPSCs, which are similar to CA1 PyrC synaptic NMDARs, may indicate that NMDARs underpin similar roles in terms of dendritic integration in PV INs (Branco et al., 2010; Comford et al., 2019). While SST INs share a common developmental origin with PV INs (Chittajallu et al., 2013; Tricoire et al., 2011) and also co-express PV (Booker et al., 2018; Jinno and Kosaka, 2000) our data indicate that through their differentiation they express functional NMDAR-EPSCs that are distinct from that of PyrCs and other PV IN morphotypes. As the single-channel conductance of NMDARs in OLM cells is similar to that of CA1 PyrCs (Hajos et al., 2002) and their dendritic filtering is rapid (Martina et al., 2000) and similar to PV INs (Nörenberg et al., 2010), our data indicate there are fundamental differences in the relative contribution of NMDARs subunits to EPSCs in these cells. In particular, it is plausible that SST INs possess a more uniform population of GluN2A/2B triheteromeric receptors, first due to the ~30% block with ifenprodil (Hansen et al., 2014) and the longer decay kinetics of NMDAR-EPSCs we observe in the GluN2A-null rats. It has been shown in mice that GluN2D is expressed in PV and SST INs in adult mice (Engelhardt et al., 2015; Standaert et al., 1996), however given the rapid kinetics and high sensitivity of NMDAR-EPSCs to GluN2A loss and ifenprodil, our data suggest that this NMDAR subunit may not contribute to synaptic currents in older rats. Indeed, it is plausible that GluN2D is expressed in rat neurons, but may contribute to non-synaptic mechanisms. As LTP in putative SST INs is dependent on NMDARs (Ouardouz and Lacaille, 1995), these receptors likely fulfil the typical role of NMDARs in LTP induction (Berberich et al., 2005) in this fundamental feedback interneuron subtype. One major consideration to the function of NMDARs in INs is that GluN2A subunits undergo a developmental increase in expression in PyrCs and INs alike (Flint et al., 1997; Martel et al., 2009; Matta et al., 2013; McKay et al., 2012; Wyllie et al., 2013). How this contributes to the circuit recruitment of INs has not been fully explored other than in a few IN subtypes (Matta et al., 2013). Indeed, whether or not GluN2D subunits undergo such a developmental trajectory in hippocampal INs, or indeed whether GluN2A subunits possess a delayed maturation in CCK INs remains unexplored.

We observed NMDAR-mediated currents in identified CCK IN cell types that were larger and slower than those observed in CA1 PyrCs. This is in good agreement with the presence of GluN2B/2D NMDARs, as described in str. radiatum INs (Perszyk et al., 2016; Yi et al., 2019) and GluN2D-containing receptors more generally (Brothwell et al., 2008; Jones and Gibb, 2005; Logan et al., 2007; Misra et al., 2000; Wyllie et al., 1996). The sensitivity of NMDAR-EPSCs in CCK INs to NAB-14 confirms the presence of GluN2C/D receptor subunits in synaptic receptors,
with a reduced sensitivity to Ifenprodil (Yi et al., 2019), compared to GluN2A/2B triheteromeric receptors. However, as GluN2C is not present in the hippocampus, the predominant receptor target is likely GluN2D-containing NMDARs. The absence of GluN2A mediated EPSC effects are in good agreement with recordings performed in non-identified str. radiatum INs, where a glycine sensitive GluN2A antagonist did not alter NMDAR-EPSCs (Yi et al., 2019). Given that LTP was strongly attenuated by NAB-14 and the lack of CP-AMPAR mediated LTP (Szabo et al., 2012) in identified CCK INs, our results support previous findings suggesting a central role of NMDARs in synaptic plasticity in these INs (Lamsa et al., 2007). Similarly, as LTP was readily induced in CCK-INs, we confirm previous work showing that GluN2D containing NMDARs aid this induction (Hrabetova et al., 2000). Together, these data support the idea that GluN2A-containing NMDARs are not required for the induction of LTP in CCK INs (Berberich et al., 2005), but rather GluN2D-containing NMDARs contribute for LTP expression.

Overall, our data extend the previous findings of Perszyk et al. (2016), where the reported mRNA corresponding to GluN2A, 2B and 2D was abundant in each of the neurochemical cell types we have assessed. For example, this study shows that GluN2A receptor subunits do not appear to contribute to synaptic NMDAR-mediated currents in CCK-INs. Whilst, our data do not preclude the possibility that other NMDAR subtypes exist in all the neuron classes tested, they indicate that such roles would be confined to other (non-synaptic or non-ionotropic) functions, such as extrasynaptic modulation of plasticity (Ivanov et al., 2006), metabotropic modulation of inhibitory receptors (Chalifoux and Carter, 2010; Guetg et al., 2010), or mediating tonic excitation (Hanson et al., 2019). Furthermore, as we have only examined the major synaptic inputs to different classes of CA1 PyrCs and INs, synapse specific effects of receptor composition may exist (Arrigoni and Greene, 2004; von Engelhardt et al., 2008). Indeed, recent data suggest that cell-type specific inputs may lead to within-cell differences in synaptic composition of NMDARs (Lei and McBain, 2002). Our data suggest that most synaptic NMDARs contain a mixed contribution of receptor subtypes, which provides the tantalising prospect that synapse and extrasynaptic heterogeneity of NMDARs exists to give rise to functional divergence. The data we present suggest that further work is required to disentangle the contribution of different NMDAR subtypes to synaptic and extrasynaptic functions in hippocampal INs.

Functional ramifications:
Given the large heterogeneity in GluN2 subunit specific effects on NMDAR-EPSCs in hippocampal INs, there are a wide variety of potential ramifications for human disease. First, given that GluN2A subunits contribute significantly to PV and SST IN synaptic NMDARs, it emphasises the need to further elucidate the role of these neurons in epileptic syndromes, as the GluN2A subunit may be a critical determinant of some forms of epilepsy (Marwick et al., 2019). Furthermore, as GluN2D de novo mutations have also been linked to epileptic encephalopathies (Camp and Yuan, 2019; Li et al., 2016) and given this subunit’s clear role in CCK IN synaptic function, this may explain the selective loss of these neurons in some forms of experimental epilepsy (Sun et al., 2014; Wyeth et al., 2010). These data suggest potential therapeutic avenues by which NMDAR function is selectively modulated in a brain region and cell type specific manner. Furthermore, NMDARs contribute to the spike coupling of INs to ongoing synaptic activity (Matta et al., 2013). How divergent NMDAR kinetic properties between different IN subtypes contributes to ongoing circuit activity remains unknown, but will likely have direct ramifications for local information processing.

In summary, we provide clear evidence for GluN2A, GluN2B and GluN2D subunit containing NMDARs in neurochemically and morphologically defined INs and PyrCs of the rat. These findings provide insight into the mechanisms of synaptic plasticity generation in CCK INs, which rely on composed of GluN2B- and GluN2D-, but not GluN2A-containing NMDARs. These data are important to our understanding of how different IN classes integrate synaptic information.
**Figure legends:**

**FIGURE 1** Absence of GluN2A confers slowed NMDAR kinetics on CA1 PyrCs. A reconstruction of a CA1 PyrC showing orientation with respect to the hippocampal layers str. oriens (Ori.), pyramidale (Pyr.), radiatum (Rad.) or lacunosum-moleculare (L-M). SOMatodendritic axis (black) and axonal arborisation (red) are indicated. B Representative EPSCs recorded from CA1 PyrCs from wild-type (black) and GluN2A-null (red) rats. AMPAR-EPSCs (inward currents) and NMDAR-EPSCs (outward currents) are shown. The peak scaled NMDAR traces are shown below to indicate the slowing of the NMDAR-EPSC. C NMDAR-EPSC rise-times, quantified for all recorded CA1 PyrCs. Data from individual cells is shown overlain as filled circles from wild-type (black, n=20 cells; N=13 rats) and GluN2A-null (2A-null; red; n=21 cells; N=11 rats). D NMDAR-EPSC decay time-constants (tau), from the weighted tau of a biexponential curve-fit. E NMDAR/AMPAR ratio of EPSCs elicited by Schaffer-collateral stimulation. Fewer rats are shown for kinetic values due to the 20 pA cut-off imposed on kinetic data. Data are shown as mean ± SD, alongside the difference between the means ± CI. Statistics shown: * - p<0.05, Student’s t-test.

**FIGURE 2:** GluN2A subunits contribute to NMDAR-EPSCs in identified PV INs. A reconstruction of a PV IN recorded in CA1 with respect to the hippocampal layers, with somatodendritic axis indicated in black and axons localised to str. pyramidale in red. Inset, immunohistological labelling for PV (green) aligned (asterisk) to the biocytin filled somata (black and white). Scale bar: 10 μm. B Representative monosynaptic AMPAR- and NMDAR-mediated EPSCs recorded from PV INs in wild-type (black) and 2A-null (red) rats. The scaled NMDAR-mediated EPSC (bottom) indicates the slowing of response in the GluN2A-null rat line. C NMDAR-EPSC rise-times, quantified for identified PV-INs, with individual cells shown overlain (filled circles) from wild-type (black, N=14 rats) and 2A-null (red; N=13 rats), fewer rats are shown for kinetic values due to the 20 pA cut-off. D Quantification of NMDAR-mediated EPSC decay time-constants (tau). E NMDAR/AMPAR ratio of EPSCs elicited by Schaffer-Collateral stimulation. Data shown as mean ± SD. Statistics shown: * - p<0.05, Student’s t-test.

**FIGURE 3:** GluN2A is a major NMDAR subunit in SSt INs. A reconstructed CA1 SSt IN shown with respect to the hippocampal layers, with somatodendritic axis confined to str. oriens (black) and axons localised to str. L-M in red. Inset, immunolabelling for SSt (green) of the
biocytin filled somata (black and white). Scale bar: 10 µm. B Representative monosynaptic AMPAR- and NMDAR-mediated EPSCs recorded from SST INs in wild-type (black) and 2A-null (red) rats. The scaled NMDAR-mediated EPSC (bottom) indicates the slower response in the GluN2A-null rats. C NMDAR-EPSC rise-times, quantification in SST INs, with individual cells shown overlain (filled circles) from wild-type (black, N=14 rats) and 2A-null (red, N=12 rats), fewer rats are shown for kinetic values due to the 20 pA cut-off. D Quantification of NMDAR-mediated EPSC decay time-constants (tau). E NMDAR/AMPAR ratio of EPSCs elicited by alveus stimulation. Data are shown as mean ± SD. Statistics shown: * - p<0.05, Student’s t-test.

FIGURE 4: GluN2A does not significantly contribute to synaptic NMDAR-mediated EPSCs in CCK INs. A reconstructed CCK BC with respect to the hippocampal layers, with somatodendritic axis covering all layers (black) and axons localised to str. pyramidale (red). Inset, immunolabelling for pre-pro-CCK (green) of the IN somata (black and white). Scale bar: 10 µm. B monosynaptic AMPAR- and NMDAR-mediated EPSCs recorded from CCK INs in wild-type (black) and 2A-null (red) rats. The scaled NMDAR-mediated EPSC (bottom) indicates no change in decay times in GluN2A-null rats. C NMDAR-EPSC rise-times in CCK INs, individual cells shown overlain (filled circles) from wild-type (black, N=26 rats) and 2A-null (red; N=16 rats), fewer rats are shown for kinetic values due to the 20 pA cut-off. D Quantification of NMDAR-mediated EPSC decay time-constants. E NMDAR/AMPAR ratio of EPSCs elicited by str. radiatum stimulation. Data are shown as mean ± SD.

FIGURE 5: GluN2B-containing NMDARs differentially contribute to EPSCs in identified hippocampal neurons. A representative NMDAR-mediated EPSCs recorded at +40 mV in the presence of CNQX at (black traces) or following 10 minutes wash-in of 10 µM Ifenprodil (grey traces) from wild-type rats (WT) for the different cell types identified. B time-course of ifenprodil washin (black bar) for CA1 PyrCs (open circles), PV INs (filled black circles), SST INs (grey circles) and a subset of CCK INs (green circles), as measured as % of control EPSC per minute, compared to 100% baseline (dashed black line). C quantification of ifenprodil block over the last 2 minutes for the different cell classes identified in wild-type and GluN2A-null neurons (2A-null), number of cells tested is shown in parenthesis. D estimation plot showing the difference in ifenprodil block between wild-type and GluN2A-null cells, plotted as the difference between the means ± 95% confidence interval. Data are shown as mean ± SD. Statistics shown: * - p<0.05, paired t-tests.
FIGURE 6: NMDAR-EPSCs in CCK INs are mediated by GluN2D, which is required for LTP induction. A representative NMDAR-mediated EPSCs recorded in CCK INs at +40 mV in the presence of CNQX at (black and red traces) or following 10 minutes wash-in of the GluN2D negative allosteric modulator NAB-14 (10 µM; grey and pink traces). B plot of NMDAR-EPSC amplitude before and after NAB-14 application in CCK INs. C quantification of NMDAR-EPSC amplitude in CCK INs from GluN2A-null rats before and after NAB-14 application. D time-course of EPSP amplitude in CCK INs from wild-type rats measured from -70 mV current-clamp, following 2x 100 Hz stimulation (double arrow) measured under control conditions (black circles) and in the presence of 10 µM NAB-14 (grey circles). Representative traces are shown above the chart, showing baseline EPSP (black traces) and 25 minutes after LTP induction (grey traces). E quantification of LTP, as reported as % change in EPSP amplitude from baseline (dashed line) for wild-type CCK INs. Control LTP recordings (black circles, n=9 cells; N=7 rats) and those performed in the present of 10 µM NAB-14 (grey circles, n=5 cells; N=5 rats) are shown. Number of tested rats shown in parenthesis. F LTP induction in CCK INs from GluN2A-null rats according to the same scheme as D. Data are shown for control recordings (red circles) and in the presence of NAB-14 (pink circles). Traces of each treatment are above the chart showing baseline (red) and after LTP induction (pink traces). G quantification of LTP induction in GluN2A-null rats under control (red, n=6 cells; N=5 rats) and in the presence of NAB-14 (pink, n=5 cells; N=4 rats). Data are shown as mean ± SD. Statistics shown: * - p<0.05, ** - p<0.01, from paired (B) and unpaired (D, F) Student’s t-test.
References


containing NMDA receptors by ambient glutamate facilitates cortical interneuron maturation.


