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14. ABSTRACT
Excessive N-methyl-D-aspartate receptor (NMDAR) activation is thought to contribute to brain diseases including epilepsy. However, we showed previously that electrographic seizures involving dentate granule cells induced by a GABAAR antagonist (BMI, 10 μM) or removal of Mg2+ (0 mM) were significantly increased following chronic D-APV treatment and modestly increased following chronic memantine treatment. Conversely BMI- and 0 mM Mg2+-induced seizures were dramatically reduced following chronic Ro25,6981 treatment. The goal of this study was to investigate potential mechanisms underlying the differential effects of distinct classes of NMDAR antagonists on seizures. To accomplish this goal, whole-cell electrophysiological recordings and morphological techniques were utilized to examine changes in synaptic connectivity in granule cells from hippocampal slice cultures treated with different classes of NMDAR antagonists.

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Shuijin He 09/07/2010

Graduate Program in Neuroscience

Uniformed Services University of the Health Sciences

ABSTRACT

Title of Thesis: Differential alterations in excitatory and inhibitory networks involving dentate granule cells following chronic treatment with distinct classes of NMDAR antagonists in hippocampal slice cultures

Name: Shuijin He

Thesis directed by: Suzanne B. Bausch
Associate Professor, Department of Pharmacology; Graduate Program in Neuroscience

Excessive *N*-methyl-D-aspartate receptor (NMDAR) activation is thought to contribute to brain diseases including epilepsy. However, we showed previously that electrographic seizures involving dentate granule cells induced by a $GABA_AR$ antagonist (BMI, 10 μM) or removal of Mg^{2+} (0 mM) were significantly increased following chronic D-APV treatment and modestly increased following chronic memantine treatment. Conversely, BMI- and 0 mM Mg^{2+}-induced seizures were dramatically reduced following chronic Ro25,6981 treatment. The goal of this study was to investigate potential mechanisms underlying the differential effects of distinct classes of NMDAR antagonists on seizures. To accomplish this goal, whole-cell electrophysiological recordings and morphological techniques were utilized to examine changes in synaptic connectivity in granule cells from hippocampal slice cultures treated with different classes of NMDAR antagonists.

In excitatory circuits onto granule cells, we found significantly reduced large amplitude sEPSC frequency following chronic Ro25,6981 treatment and a trend toward increased large amplitude sEPSC duration following chronic D-APV treatment, consistent with our previous report describing opposite effects of chronic treatment with D-APV and with Ro25,6981 on pathological hyperactivity. We also found increased mEPSC and $mEPSC_{AMPAR}$ frequency and amplitude following chronic treatment with D-APV,

memantine and Ro25,6981, and increased vGlut1-positive synaptic contacts onto granule cell dendrites following chronic treatment with all NMDAR antagonists except Ro25,6981. However, changes in individual glutamatergic synaptic connectivity and synaptic number could not account for altered neuronal network excitability following chronic NMDAR antagonist treatments.

In inhibitory circuits, sIPSC frequency was dramatically reduced in both granule cells and hilar interneurons only following chronic Ro25,6981 treatment. No significant alteration in mIPSC frequency or amplitude implicated diminished action potential-dependent GABA release. Electrophysiological recordings suggested that the reduced action potential-dependent GABA release could not be attributed to altered action potential or membrane properties, altered GABA release probability or failure rate in interneurons. However, occlusion of chronic Ro25,6981-mediated decreases in sIPSCs by acute application of specific Kv1 blockers, suggested that functional up-regulation of Kv1 channels played a prominent role in diminished action potential-dependent GABA release.

Taken together, these homeostatic changes in glutamatergic and GABAergic network activities may shed light on the therapeutic utilization of Ro25,6981 to treat epilepsy.

Differential alterations in excitatory and inhibitory networks involving dentate granule cells following chronic treatment with distinct classes of NMDAR antagonists in hippocampal slice cultures

by

Shuijin He

Doctoral Dissertation submitted to the faculty of Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2010

Thesis directed by:
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最后，谨以此文献给我的已逝去的哥哥，希望他在天之灵安息。

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LIST OF ABBREVIATIONS:

4-AP: 4-aminopyridine

AHP: after hyperpolarization potential

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AP: action potential

BMI: bicuculline methiodide

BFNC: benign familiar neonatal convulsions

CA1: cornu ammonis field 1

CA2: cornu ammonis field 2

CA3: cornu ammonis field 3

cAMP: cyclic adenosine monophosphate

CCK: cholecystokinin

cGMP: cyclic guanosine monophosphate

CGP55845: (2S)-[[(1S)-1-(3,4-Dichlorophenyl)ethyl] amino-2-hydroxypropyl](phenylmethyl)phosphinic acid

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CPPG: RS-α-cyclopropyl-4-phosphophenylglycine

D-APV: D(-)-2-amino-5-phosphonopentanoic Acid

DG: dentate gyrus

D/H border interneuron: interneuron located in the dentate/hilus border zone

DIV: days *in vitro*

DL-TBOA: D,L-threo-β-benzyloxyaspartate

DNQX: 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide disodium salt

eIPSC: evoked post-synaptic current

EDTA: ethylenediaminetetraacetic acid

EPSC: excitatory post-synaptic current

ERK: extracellular signal-regualted kinase

fAHP: fast afterhyperpolarization potential

GABA: gamma-aminobutyric acid

$GABA_AR$: gamma-aminobutyric acid A receptor

$GABA_BR$: gamma-aminobutyric acid B receptor

GIRK channel: G-protein activated inward rectifier potassium channel

GPCR: G-protein coupled receptor

HEPES: 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid;

HICAP cell: hilar commissural-associational associated interneuron

HIPP cell: hilar perforant pathway-associated interneuron

IPSC: inhibitory postsynaptic current

KAR: kainate receptor

Kir channel: inward rectifier potassium channel

Kv channel: voltage-gated potassium channel

LTD: long term depression

LTP: long term potentiation

MAP2: microtubule associated protein 2

MAGUKs: membrane-associated guanylate kinases

mEPSC: miniature excitatory post synaptic current

$mEPSC_{AMPAR}$: AMPAR-mediated mEPSC

$mEPSC_{NMDAR}$: NMDAR-mediated mEPSC

mGluR: metabotropic glutamate receptor

mIPSC: miniature inhibitory post synaptic current

mPSC: miniature postsynaptic current

MRS2365: [(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid monoester

MRS2500: (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]- 2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate receptor

NPY: neuropeptide-Y

PPR: paired pulse ratio

PSC: postsynaptic current

R_{IN}: input resistance

RMP: resting membrane potential

sEPSC: spontaneous excitatory postsynaptic current

$sEPSC_{large:}$ large amplitude spontaneous excitatory postsynaptic current

$sEPSC_{small}$: small amplitude spontaneous excitatory postsynaptic current

sIPSC: spontaneous inhibitory postsynaptic current

SIC: NMDAR-mediated slow inward current

SynGAP: synaptic Ras-GTPase activating protein

TBI: traumatic brain injury

TeNT: tetanus toxin

TLE: temporal lobe epilepsy

TEA: tetraethylammonium

TTX: tetrodotoxin

vGlu1: vesicular glutamate transporter 1

VIP: vasoactive intestinal peptide

XE 991: 10,10-bis(4-Pyridinylmethyl)-9(10*H*)-anthracenone dihydrochloride

List of compounds and their pharmacological actions

Name of compound	Pharmacological action
4-AP	Kv channel blocker
BMI	$GABA_A$ receptor antagonist
CGP55845	$GABA_B$ receptor antagonist
CPPG	group III mGlu receptor antagonist
CNQX	kainate/AMPA receptor antagonist
D-APV	high-affinity competitive NMDAR antagonist
dendrotoxin	Kv1.1-, Kv1.2- and / or Kv1.6-containing channel blocker
dendrotoxin-K	specific Kv1.1-containing channel blocker
DL-TBOA	glutamate transporter blocker
DNQX	AMPA receptor antagonist
GABA	GABA receptor agonist
margatoxin	specific Kv1.3 blocker
memantine	moderate-affinity uncompetitive NMDAR antagonist
MK-801	high-affinity uncompetitive NMDAR antagonist
MRS2365	P2Y1 receptor agonist
MRS2500	P2Y1 receptor antagonist
NMDA	NMDAR agonist
NVP-AAM077	NR2A-containing NMDAR antagonist
Ro25,6981	NR2B-containing NMDAR antagonist
QX-314	non membrane permeable sodium channel blocker
TEA	Kv channel blocker

List of compounds and their pharmacological actions

Name of compound	Pharmacological action
TeNT	specific neurotransmitter release blocker by cleaving synaptobrevin
TTX	extracellular sodium channel blocker
XE 991	specific Kv7 channel blocker

CHAPTER 1

General Introduction

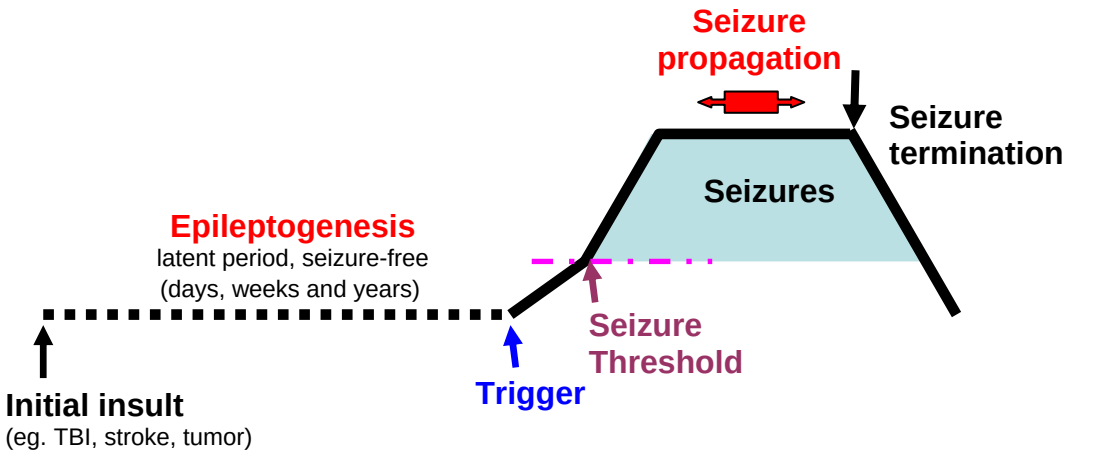
Temporal lobe epilepsy (TLE) is a treatable neurological disorder.

Epilepsy is one of the most common chronic neurological disorders characterized by recurrent unprovoked seizures. It affects about 1% of the population (Shin and McNamara, 1994) and currently, around 2.7 million people in the US are suffering from epilepsy (Begley et al., 2000). Epilepsy consists of 40 different forms, which are often classified by two different criteria. Firstly, epilepsy can be categorized by etiology as idiopathic epilepsy, cryptogenic epilepsy or symptomatic epilepsy. They arise from genetic abnormalities, unidentified prenatal structural lesions and known postnatally acquired lesions, respectively. Secondly, epilepsy can be classified into partial epilepsy, generalized epilepsy and epilepsy of unknown localization according to location of seizure onset and distribution of seizures. Partial (focal, local) epilepsy is a term referring to seizures arising from and restricted to a portion of one cerebral hemisphere, and may become secondarily generalized. Generalized epilepsy, in contrast, refers to epileptic seizures involving many foci in both hemispheres simultaneously (Dreifuss, 1997).

Temporal lobe epilepsy (TLE) is a form of partial epilepsy that originates from temporal lobe and is the most common type of epilepsy. TLE accounts for ~30% of all epilepsies (Spencer et al., 1982; Cahan et al., 1984). TLE is a symptomatic epilepsy due to its etiology. Like other symptomatic epilepsies, TLE can be acquired by brain insults such as head injury, stroke, trauma, infections or malignancies. Currently, treatments for

TLE are not satisfactory because 30% of TLE patients are resistant to antiseizure drugs (Shin and McNamara, 1994). The currently available antiseizure drugs prevent the occurrence of seizures (symptom) but cannot prevent epileptogenesis, which is the process that leads to the development of chronic recurrent spontaneous seizures (epilepsy). The process is often associated with a seizure-free “silent” or “latent” period that can last days, months or even years following brain insults (Fig. 1). During epileptogenesis, changes that often occur in the structure and physiology of the brain subsequently lead to hyperexcitability in neural networks. Thus, our hope for curing epilepsy is to develop drugs that prevent epileptogenesis in the brain following insults. Exploring the mechanisms contributing to epileptogenesis is the first step toward success in developing a new drug for preventing epileptogenesis (Bausch, 2008).

The temporal lobe is a region of the cerebral cortex that is located anterior to the occipital cortex and inferior to the Sylvian fissure. The temporal lobe contains the primary auditory cortex, the amygdala, the hippocampus, and the parahippocampal gyrus. According to ILAE classification, there are two main types of temporal lobe epilepsies, lateral TLE and mesial TLE. While lateral TLE originates in the neocortex on the outer surface of the temporal lobe, mesial TLE arises in the hippocampus, the parahippocampal gyrus and the amygdala, which are located beneath outer surface neocortex. Mesial TLE is the major form of TLE associated with hippocampal and amygdala sclerosis.



Modified from Bausch SB / Schwartzkroin PA

Figure 1. The process of epileptogenesis. Brain insults such as traumatic brain injury (TBI), stroke, tumor can initiate epileptogenesis, which is the process by which a brain becomes chronically prone to seizures. This process is associated with a seizure-free period that can last days, weeks or even years. Seizures occur once a factor (eg. alcohol, stress, diet, infection) triggers or unmasks underlying brain hyperexcitability and reaches seizure threshold. After that, seizures can propagate in the brain and eventually terminate. Current antiseizure drugs target seizure threshold and propagation.

Role of hippocampus and dentate gyrus in TLE

The hippocampal formation is divided into three cytoarchitectonic regions: the dentate gyrus; the hippocampus composed of the CA3, CA2 and CA1 fields (Fig. 2); and the subiculum (Lorente de Nó, 1933; Lorente de Nó, 1934; Blackstad, 1956; Amaral, 1978). The perforant pathway that originates from the entorhinal cortex is the main extrinsic input into the hippocampal formation from the cortex and provides predominant excitatory inputs onto the dentate granule cells from the cerebral cortex. Within the hippocampal formations on a very simple level, a trisynaptic unidirectional connection is formed: dentate granule cells→CA3 pyramidal cells→CA1 pyramidal cells (Fig 2). The hippocampus is one of the most seizure prone structures in the brain, in which CA3 is the most vulnerable region to seizures (Nadler et al., 1978).

Hippocampal sclerosis is a hallmark of TLE human patients (Babb et al., 1984b; Babb et al., 1984a) and animal models including kainic acid (Nadler, 1981) and pilocarpine (Turski et al., 1983), and is characterized by a selective loss of neurons in the hilus, CA3, and CA1, but sparely in the CA2 field (Margerison and Corsellis, 1966; Bruton, 1988). Surgical resection of the sclerotic hippocampus has been shown to cure epilepsy in selected patients with TLE (Spencer et al., 1982; Dodrill et al., 1986; Walczak et al., 1990), suggesting that the hippocampus plays a crucial role in the occurrence and propagation of temporal lobe seizures.

The dentate gyrus consists of three cytoarchitectonic layers: the molecular layer; the granule cell layer; and the hilus, which is also called the polymorphic region composed of a variety of types of mossy cells and interneurons. The dentate gyrus is thought to be a filter/gate for seizure propagation from the entorhinal cortex to the hippocampus because

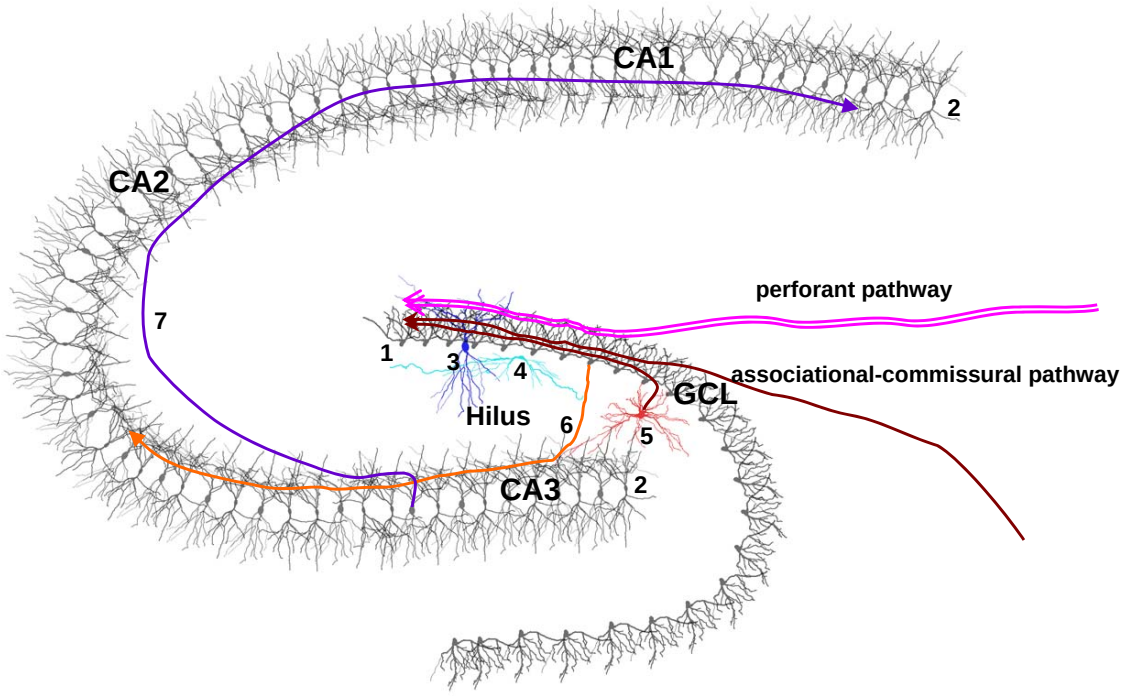


Figure 2. Hippocampal structure and pathways. The hippocampal formation consists of the dentate gyrus, the hippocampus proper, which is composed of the CA3, CA2 and CA1 fields, and the subiculum (Lorente de Nó, 1933; Lorente de Nó, 1934; Blackstad, 1956; Amaral, 1978). There are two main excitatory inputs onto dentate granule cells. While the perforant pathway originates from the entorhinal cortex and innervates dendrites of granule cells in the outer 2/3 of the molecular layer, the associational-commissural pathway arises from ipsilateral and contralateral mossy cells and terminates in the inner 1/3 of the molecular layer. Within the hippocampal formation, a trisynaptic unidirectional connection is formed: dentate granule cells→CA3 pyramidal cells→CA1 pyramidal cells. Mossy fibers convey information from granule cells to CA3 pyramidal cells as well as hilar interneurons. Schaffer collaterals are the axons of CA3 pyramidal cells, which mainly innervate CA1 pyramidal cells. **1**) granule cell; **2**) pyramidal cell; **3**) high frequency firing interneuron (axo-axonic cell and basket cell); **4**) adapting firing interneuron (HICAP, HIPP and everywhere cells); **5**) mossy cell; **6**) mossy fiber; **7**) Schaffer collateral.

of the unique characteristics of granule cells: (1) specific intrinsic electrical membrane properties (Mody et al., 1988; Staley et al., 1992; Jung and McNaughton, 1993); (2) low incidence of recurrent excitatory synapses between granule cells (Molnar and Nadler, 1999; Okazaki et al., 1999); and (3) strong inhibitory inputs onto granule cells from hilar interneurons under normal physiological conditions (Ribak and Anderson, 1980; Ribak and Peterson, 1991; Halasy and Somogyi, 1993b; Han et al., 1993), but disrupted or impaired in TLE (Dam, 1980; Sloviter, 1987; Babb et al., 1989; Obenaus et al., 1993). Neuronal hyperexcitability in the entorhinal cortex goes through the dentate gyrus before it propagates into the CA3 region. The perforant pathway conveys information from the entorhinal cortex to the dentate gyrus and terminates in the outer 2/3 of the molecular layer. In addition to the perforant pathway, granule cells also receive intrahippocampal excitatory inputs via the associational/commissural pathway (Fig 2). It arises from the ipsilateral and contralateral mossy cells (Buckmaster et al., 1996; Freund and Buzsaki, 1996; Wenzel et al., 1997), which are glutamatergic neurons (Frotscher et al., 1991; Wenzel et al., 1997) and constitute about 50% of hilar interneurons, and terminate in the inner 1/3 of the molecular layer.

Intrinsic membrane properties of dentate granule cells

Intrinsic membrane properties consist of input resistance, resting membrane potential and capacitance as well as action potential threshold, frequency, amplitude and half-width. Compared with hippocampal pyramidal neurons, dentate granule cells have a relatively more negative resting membrane potential and exhibit low-frequency firing (Staley et al., 1992; Jung and McNaughton, 1993) that can limit neuronal hyperexcitability. Alterations

in electrical membrane properties may be one cause of impairment of the filter function of granule cells. For example, a higher input resistance reduces synaptic shunting, whereas a more negative action potential threshold facilitates neuronal firing. As a result, they both can increase neuronal excitability. Indeed, several previous studies reported increased action potential duration and peak amplitude in a subset of granule cells from resected hippocampal tissues of epileptic patients (Dietrich et al., 1999) and increased input resistance in rat hippocampal granule cells following electrical kindling of an animal model of epileptogenesis (Mody et al., 1988). However, a number of studies showed no significant difference in membrane properties between epileptic and control granule cells (Isokawa et al., 1991; Williamson et al., 1993; St John et al., 1997). Thus, the contribution of changes in intrinsic electrical membrane properties to TLE remains controversial.

Glutamatergic transmission and mossy fiber sprouting in dentate granule cells

Glutamate is the major excitatory neurotransmitter that can activate ionotropic AMPA, kainate and NMDA receptors and metabotropic glutamate (mGlu) receptors. Ionotropic glutamate receptors mediate fast excitatory synaptic transmission and play a pivotal role in neuronal excitability, while mGlu receptors are G-protein coupled receptors that modulate K^+ and Ca^{2+} channel function as well as the level of cytoplasmic cAMP and cGMP (Bockaert et al., 2002). Thus, changes in glutamatergic transmission onto granule cells could directly drive the dentate gyrus toward hyperexcitability, impair its filter function and allow seizure propagation into the hippocampus. Numerous studies showed that changes in glutamatergic transmission are associated with the development of TLE.

A massive loss of glutamatergic mossy cells is seen in TLE human patients (Blumcke et al., 2000; Loup et al., 2000) and animal models of epilepsy (Scharfman et al., 2001), but the functional consequence is still under debate. More importantly, documented changes in glutamatergic transmission associated with TLE include: (1) altered NMDAR properties in dentate granule cells exhibiting increased mean open time and reduced Mg^{2+} block (Kohr et al., 1993), (2) upregulated NMDAR and AMPAR (Yeh et al., 1989; Ullal et al., 2005), and (3) elevated extracellular levels of glutamate in epileptic hippocampus (Benveniste et al., 1984).

Mossy fiber sprouting is one of the most frequently observed features in the epileptic hippocampal formation from TLE animal models (Tauck and Nadler, 1985; Sutula et al., 1988; Mello et al., 1992) and humans (de Lanerolle et al., 1989; Sutula et al., 1989; Houser et al., 1990). Mossy fibers are the axons of granule cells that normally form glutamatergic synapses onto CA3 pyramidal cells as well as hilar interneurons. Mossy fiber sprouting is the abnormal ingrowth of mossy fibers collaterals into the inner molecular layer, where there are few mossy fiber collaterals under normal conditions (Sutula et al., 1989). Mossy fiber sprouting can be detected with Timm staining due to high zinc content in mossy fibers (Ibata and Otsuka, 1969). Sprouted mossy fibers form new recurrent excitatory synapses predominantly onto granule cells (Sutula et al., 1988; Sutula et al., 1989; Represa et al., 1993; Buckmaster et al., 2002), but to a lesser degree onto interneurons (Ribak and Peterson, 1991). Giant EPSPs could be detected in the molecular layer of the epileptic hippocampi following antidromic stimulation of the hilus (Tauck and Nadler, 1985; Cronin et al., 1992; Okazaki et al., 1999; Lynch and Sutula, 2000; Williams et al., 2002), which has been attributed to mossy fiber sprouting.

However, the contribution of mossy fiber sprouting to epileptogenesis and seizures remains controversial because mossy fiber sprouting also increases excitatory inputs onto GABAergic interneurons and prevention of mossy fiber growth by cycloheximide had no effect on the development and severity of spontaneous seizures in the pilocarpine and kainic acid animal model (Longo and Mello, 1997, 1998). Subsequently, another group showed that cycloheximide did not impact mossy fiber growth in the pilocarpine animal model (Williams et al., 2002). Thus, further studies are needed to elucidate this discrepancy.

GABAergic transmission onto dentate granule cells

GABA is the major inhibitory neurotransmitter in the brain and acts on $GABA_A$ and $GABA_B$ receptors. The $GABA_B$ receptor is a G-protein coupled receptor that can activate presynaptically G-protein gated inward rectifier potassium (GIRK) channels, postsynaptically activate calcium channel function and reduce levels of intracellular cAMP (Klix and Bettler, 2002). The $GABA_A$ receptor is a ligand gated ion channel composed of five subunits, with $\alpha_2\beta_2\gamma$ being the most predominant combination in the brain (Olsen and Macdonald, 2002). $GABA_A$ receptors mediate fast synaptic transmission through the flow of negatively charged chloride ions and are responsible for the majority of GABAergic innervation in the brain. Loss of hilar GABAergic interneurons is a frequently observed and important feature of patients and animal models of TLE (Dam, 1980; Sloviter, 1987; Babb et al., 1989; Obenaus et al., 1993).

GABAergic interneurons are a very diverse subpopulation of neurons in the brain and their classification continues to be a subject of much debate (Maccaferri and Lacaille,

2003; Monyer and Markram, 2004). One way of classifying interneurons is according axonal projections and firing properties. For example in Chapter 3, hilar interneurons were classified into hilar commissural-associational associated cells (HICAP cells), hilar perforant pathway associated cells (HIPP cells), axo-axonic cells and basket cells (Fig 3) based upon their axonal distribution and firing patterns as described previously (Han et al., 1993; Freund and Buzsaki, 1996) and in the Chapter 3. Briefly, axo-axonic cells were coined by Somogyi (1977) according to their projections onto axonal initial segments of postsynaptic neurons and are characterized by axonal arborizations predominantly in the granule cell and the CA3c pyramidal cell layers and chandelier-like rows of boutons. Basket cells are characterized by axons almost entirely restricted to the granule cell layer with net-like boutons surrounding granule cells. HICAP cells are characterized by axonal collaterals distributed predominantly in the outer granule cell layer and the inner 1/3 of the molecular layer. HIPP cells are characterized by axonal collaterals distributed predominantly in the outer 2/3 of the molecular layer.

Another classification scheme for GABAergic interneurons has emerged according to expression of neuropeptides and/or calcium-binding proteins (Freund and Buzsaki, 1996). The hilar interneurons mainly contain three types of calcium-binding proteins: parvalbumin, calbindin and calretinin; and four types of neuropeptides: somatostatin, cholecystokinin (CCK), vasoactive intestinal peptide (VIP) and neuropeptide-Y (NPY). These calcium-binding proteins and neuropeptide content also can be colocalized in a single GABAergic interneuron. For example, VIP is colocalized with calretinin or CCK in some interneurons (Freund and Buzsaki, 1996). Meanwhile, this classification of interneurons according to calcium-binding proteins or peptides shows an overlap with the

four classic interneuron types characterized by axonal distribution. All parvalbumin-containing interneurons are axo-axonic and/or basket cells, but cannot account for all axo-axonic and basket cells (Ribak et al., 1990). A subset of basket cells contains CCK and/or VIP instead of parvalbumin (Nunzi et al., 1985). The majority of somatostatin-containing interneurons have axons predominantly distributed in the molecule layer of the dentate gyrus (Bakst et al., 1986; Buckmaster and Jongen-Relo, 1999), which are also classified as HIPP cells

The somatostatin-containing interneurons account for about 30% of all hilar GABAergic interneurons (Kosaka et al., 1988) and approximately 83% of these hilar interneurons are lost following kainate treatment in rats (Sloviter, 1987). In contrast, the dentate/hilar (D/H) border interneurons, many of which are basket or axo-axonic cells, survive in TLE (Robbins et al., 1991; Obenaus et al., 1993) and provide strong inhibition onto dentate granule cells. Thus, study of interneurons is important for understanding the mechanisms underlying epileptogenesis and the subsequent expression of seizures.

GABAergic inhibition plays a dual role in brain function and possibly seizure occurrence through balancing excitation and synchronizing neuronal firing. An increase in GABAergic inhibition can reduce neuronal excitability through shifting the balance between excitation and inhibition toward inhibition. On the other hand, an increase in GABAergic inhibition is thought to promote neuronal synchronization. A primary and as yet incompletely answered question is how these two functions (balancing excitation and synchronizing neuronal firing) collectively modulate excitability. GABAergic inhibition includes hyperpolarizing inhibition and shunting inhibition. Hyperpolarizing inhibition makes the membrane potential more negative, resets the onset of spikes to a precise time

and produces “rebound” excitation by activating hyperpolarization-gated channels (Andersen and Eccles, 1962; Cobb et al., 1995; Mann and Paulsen, 2007). By contrast, shunting inhibition increases membrane conductance and facilitates synchronous firing through minimizing synaptic inputs and cessation of spike firing in a short time window (Mann and Paulsen, 2007). Thus, hyperpolarization and shunting inhibition work together to tune the precise timing of spike generation and produce the rhythmic synchronous firing in a population of neurons (Cobb et al, 1995), which is a key for spontaneous seizure occurrence.

The mechanisms underlying the dual role of GABAergic inhibition in seizure occurrence are still under debate (Freund, 2003; Mott and Dingledine, 2003). The dual role of GABAergic inhibition in seizures may be also dependent upon the specific subcellular membrane domains where GABAergic synapses are formed. Perisomatic inhibition, which includes GABAergic synapses onto axonal initial segments, somata, proximal dendrites, is mainly responsible for neuronal synchronization while dendritic inhibition appears more suited to maintain the balance between excitation and inhibition (Freund, 2003). Axo-axonic and basket cells that form perisomatic inhibition are the most likely candidates for tuning spike timing precisely because: 1) one axo-axonic cell or basket cell can innervate thousands of principle cells; 2) they fire action potentials phase locked to theta oscillation (4-7 Hz) and to sharp-wave-associated ripple oscillations (120-200 Hz) in hippocampal pyramidal cells *in vivo* (Klausberger et al., 2003); 3) synaptic and electric connections are formed between basket cells (Tamas et al., 2000; Traub et al., 2001); and 4) they can fire rhythmic and high frequency action potentials without adaptation. Consistently, axo-axonic and basket cells were previously reported to act as a

generator for theta (4-7 Hz) and gamma oscillation (30-90 Hz) in hippocampal pyramidal cells (Cobb et al., 1995; Miles et al., 1996; Mann et al., 2005). Thus, an increase in GABAergic inhibition from axo-axonic and basket cells may contribute to seizure expression involving dentate granule cells due to enhanced neuronal synchronization. In contrast, an increase in GABAergic inhibition from HICAP cells and HIPP cells may suppress seizures because of reduced excitatory synaptic integration, which is the process that converts many voltage deflections caused by different spatial and temporal synaptic inputs into a single change in membrane potential (Magee, 2000).

NMDA receptors: function, structure and antagonists

The importance of NMDAR activation for synaptic plasticity and neuronal survival

The NMDA receptor is a heteromeric ligand-gated ion channel that is composed of NR1 and NR2 subunits (Fig. 3) (Dingledine et al., 1990) and in some cases contains NR3 subunit (Cull-Candy et al., 2001). NR2 subunits consist of NR2A, NR2B, NR2C and NR2D, with NR2A and NR2B being the most prevalent subunits in the hippocampus and cortex (Yamakura and Shimoji, 1999). The NR3 subunit was first identified in the rat brain in 1995 by two groups (Ciabarra et al., 1995; Sucher et al., 1995). The 7 NMDAR subunits are encoded by 7 genes, respectively: GRIN1 for NR1, GRIN2A for NR2A, GRIN2B for NR2B, GRIN2C for NR2C, GRIN2D for NR2D, GRIN3A for NR3A and GRIN3B for NR3B (Dingledine et al., 1999; Andersson et al., 2001). The NR3 subunit does not form functional NMDA receptors alone, but is able to coassemble with the NR1 subunit or the NR1/NR2 complex to form functional receptors (Chatterton et al., 2002; Cavara and Hollmann, 2008). Compared with non-NMDA ionotropic glutamate receptors

(AMPAR and kainate receptor), NMDA receptors display certain unique characteristics including relatively slow activation / deactivation kinetics, high permeability to calcium and voltage-dependent Mg^{2+} block (Dingledine et al., 1990), which are very important for the integration of synaptic activity. The inclusion of the NR3 subunit to NR2-containing NMDARs can reduce agonist-induced current amplitude, Mg^{2+} sensitivity, and calcium permeability (Cavara and Hollmann, 2008). In addition, NR1 and NR3 subunits can form a glycine receptor, which can be activated by glycine alone and is not affected by conventional NMDAR agonists and antagonists (Barth et al., 2005; Nakanishi et al., 2009).

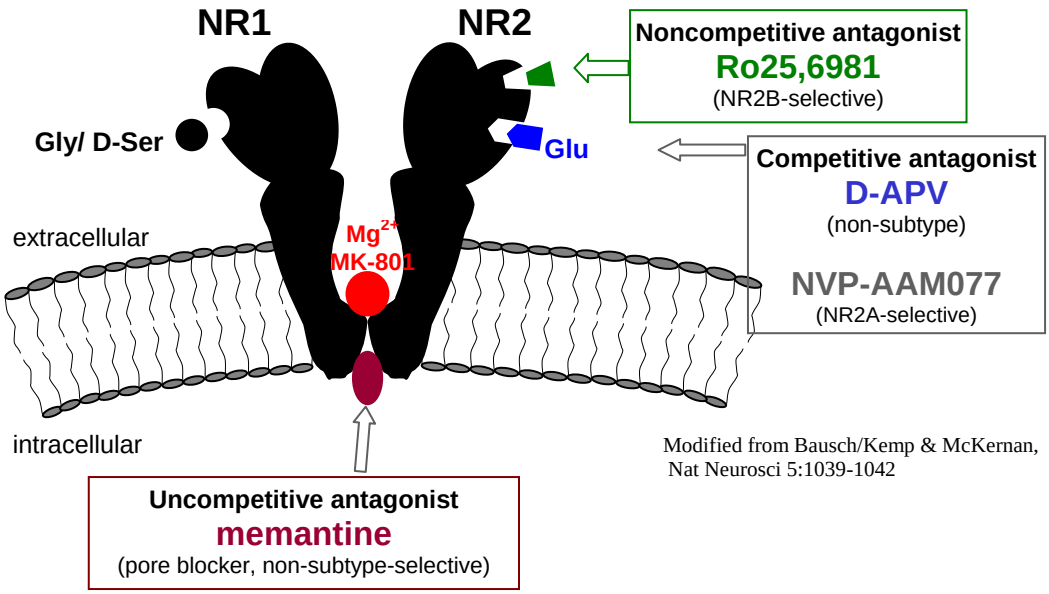


Figure 3. NMDAR structure and antagonists. The *N*-methyl-D-aspartate receptor (NMDAR) is a heteromeric ionotropic channel composed of NR1 and NR2A-D subunits, with NR2A and NR2B being the most prominent of the NR2 subunits in the hippocampus and cortex. Representative NMDAR antagonists were selected in this study based on their pharmacological properties as described in Chapter 2. D-APV (50 μM) was chosen as the high-affinity competitive selective antagonist because of its specificity for NMDAR and frequent use for complete NMDAR blockade in physiological experiments (Evans et al., 1982). Memantine (1 μM) was selected as the moderate-affinity uncompetitive antagonist because of its clinical utilization and preferential inhibition of NMDAR hyperactivation (Parsons et al., 1999). Ro25,6981 (1 μM) was chosen as the NR2B selective antagonist because of its high affinity and selectivity for NR2B-containing NMDAR (Fischer et al., 1997). NVP-AAM077 (50 nM) was selected as the NR2A selective antagonist because of its preference for NR2A-containing NMDAR at this concentration (Neyton and Paoletti, 2006).

Activation of NMDA receptors requires 3 criteria (Fig. 3): (1) an agonist (glutamate) binding to NR2 subunits (Madden, 2002); (2) a coagonist (glycine or D-serine) binding to NR1 subunits; and (3) removal of voltage-dependent Mg^{2+} block. Because of its unique properties, NMDAR is an ideal candidate to underlie the process of learning and memory. In support of this assumption, LTP and LTD, the forms of synaptic plasticity underlying leaning and memory, were abolished by NMDAR antagonists (Collingridge et al., 1983; Morris, 1989). Aside from synaptic plasticity, compelling evidence shows that NMDAR activation plays an important role in neural development and neuronal survival (Ikonomidou et al., 1999). However, excessive activation of NMDAR is thought to contribute to pathophysiology of many brain diseases such as epilepsy, Alzheimer's disease, Huntington's disease, Parkinson's disease, neuropathic pain (Dingledine et al., 1999). This led to development of NMDAR antagonists as potential therapeutic agents for treating brain diseases clinically. It is increasingly evident that antagonism of NMDAR produces protective effects against epilepsy, ischemic stroke, parkinsonism and neuropathic pain in animal models (Palmer, 2001; Parsons, 2001; Kemp and McKernan, 2002). Surprisingly, chronic complete inhibition of NMDARs paradoxically increases neuronal excitability and is associated with increased number of synapses and upregulation of NMDARs (Rao and Craig, 1997; Liao et al., 1999; Luthi et al., 2001), whereas acute withdrawal of chronic NMDAR antagonists can result in epileptic seizures (Yang et al., 2001; Wang and Bausch, 2004; Bausch et al., 2006). Moreover, complete NMDAR blockade for a few hours significantly promotes programmed cell death in the developing rat brain *in vivo* (Ikonomidou et al., 1999). Therefore, the right level of NMDAR activity is critical for maintaining normal brain function.

Differential roles of NR2A- and NR2B-containing NMDAR in synaptic plasticity and receptor trafficking

NR2A and NR2B are the two predominant NR2 subunits in the adult hippocampus and cortex (Monyer et al, 1994). NR2A subunits cannot be detected at birth but thereafter gradually increase to adult levels in the hippocampus and cortex. In contrast, the level of NR2B subunit is rather stable in the hippocampus after birth, and slightly decreases to the adult level in the cortex from the strong expression at birth (Monyer et al., 1994). During brain development, the number of NR2A subunits is inserted into synapses to replace NR2B subunits and thereby shorten the duration of the synaptic NMDAR-mediated currents because NR2A subunits bear faster kinetics than NR2B subunits (Monyer et al., 1994; Flint et al., 1997). This developmental change may lead to the NR2A subunit being predominant NR2 subunits in synaptic sites and NR2B subunits being primarily located in the extrasynaptic membrane in mature hippocampal neurons (Stocca and Vicini, 1998; Tovar and Westbrook, 1999). However, recently, Thomas et al. (2006) reported that synaptic and extrasynaptic NMDARs contain both NR2A and NR2B in cultured mature hippocampal neurons. Thus, the notion of synaptic and extrasynaptic NMDARs consisting of different NR2 subunits remains debated.

The membrane surface NR2A and NR2B subunits are dynamic and differentially regulated by activity. Insertion of NR2A-, but not NR2B-containing NMDARs into the cell-surface membrane is regulated by synaptic activity (Barria and Malinow, 2002). NR2B-containing NMDARs undergo endocytosis faster than NR2A-containing NMDARs due to different phosphorylation properties in their C-terminus (Lavezzari et al., 2004). Synaptic NMDARs are more static than extrasynaptic NMDARs because

postsynaptic scaffolding proteins, MAGUKs, protect NMDARs from clathrin-dependent internalization (Roche et al., 2001; Lavezzari et al., 2003). However, synaptic NMDARs can exchange with extrasynaptic receptors via endo-exocytic trafficking and lateral diffusion (Wenthold et al., 2003).

In addition to surface expression and receptor trafficking, NR2A- and NR2B-containing NMDARs were also reported to play differential roles in physiological functions including synaptic plasticity. Activation of NR2A-containing NMDARs was previously reported to regulate the induction of LTP in hippocampal CA1 synapses, whereas activation of NR2B-containing NMDARs was responsible for the induction of LTD (Liu et al., 2004). Subsequently, the same group found that knockdown of NR2A subunits downregulated, but blockade of NR2B subunits with selective antagonists enhanced the surface expression of AMPAR (Kim et al., 2005). These data may account for the differential role of NR2A- and NR2B-containing NMDARs in synaptic plasticity because AMPAR insertion and trafficking are thought to be a major mechanism for expression of synaptic plasticity (Lu et al., 2001; Malinow and Malenka, 2002). On other hand, other labs showed that activation of either NR2A- or NR2B-containing NMDARs could induce LTP (Berberich et al., 2005) and that overexpression of NR2B in transgenic mice could enhance LTP (Tang et al., 1999). Thus, the selective role of NR2 subunits in synaptic plasticity remains controversial.

We previously showed that chronic inhibition of NR2B-containing NMDAR dramatically reduced the expression of electrographic seizures involving dentate granule cells induced by acute blockade of $GABA_A$ receptors or acute removal of Mg^{2+} block for NMDA receptors in hippocampal slice cultures (Wang and Bausch, 2004; Dong and

Bausch, 2005). Recently, Chen et al. (2007) reported that activation of NR2A-, but not NR2B-containing NMDARs is required for the induction of epileptogenesis in animal models, a finding that is seemingly contrary to our previous data. In their studies, NMDAR antagonists were administrated acutely into rat brains prior to electrical or chemical inducing stimuli to block the induction of epileptogenesis. However, in our studies, NMDAR antagonists are applied chronically to the culture medium after the epileptogenesis inducing stimuli, which is deafferentation into the dentate gyrus and hippocampus. Hence, different paradigms for epileptogenesis induction and / or NMDAR maintenance may account for the discrepancy.

A moderate affinity uncompetitive antagonist and an NR2B subunit-selective antagonist as potential therapeutic treatment for epileptogenesis

The rationale for choosing these antagonists is described in more detail in Chapter 2 (Fig. 3). Briefly, high affinity NMDAR antagonists are not suitable for treating human patients because of many side effects including cognitive dysfunction, psychotomimetic actions, neuronal vacuolization and paradoxical seizure exacerbation in humans and animal models (Olney et al., 1989; Sveinbjornsdottir et al., 1993; Gould et al., 1994; Muir and Lees, 1995; Loscher, 1998; Ikonomidou et al., 1999; Parsons et al., 1999; Wang and Bausch, 2004). Chronic treatment with the high affinity NMDAR antagonist D-APV can cause axonal sprouting (McKinney et al., 1999; Wang and Bausch, 2004) and increased NMDAR clusters *in vitro* (Rao and Craig, 1997; Liao et al., 1999), which would increase rather than decrease neuronal excitability. Initially, the moderate-affinity uncompetitive NMDAR antagonist, memantine, was thought to be a potential therapeutic agent for

treating epilepsy because of transient use- and voltage-dependent block and fast open channel dissociation kinetics. However, subsequent studies showed that chronic treatment with memantine increased the susceptibability of seizures (Parsons et al., 1995; Wang and Bausch, 2004). Thus, only subunit-selective NMDAR antagonists hold the promise to treat temporal lobe epileptogenesis in the future (Chen et al., 1992; Muir and Lees, 1995; Kew et al., 1996). They may preferentially block pathological hyperactivity due to an allosteric as well as pH and use-dependent inhibition, and elicited fewer adverse effects and were well-tolerated in clinic trials.

Introduction to my study

An *in vitro* model of TLE

Organotypic hippocampal slice cultures

Numerous animal models of TLE have been developed to study the cellular and molecular mechanisms underlying epileptogenesis. The frequently used models include delivery of pilocarpine or kainate and inducing kindling. Pilocarpine and kainic acid activate muscarinic acetylcholine receptors and kainate receptors, respectively, whereas kindling produces temporal lobe seizures by repeated electrical stimulations of seizure prone structures, the amygdala and the hippocampus, to lower seizure threshold (see Bausch, 2008). These TLE animal models display common characteristics including cell loss, synaptic reorganization and mossy fiber sprouting and display a definable latent period except for the kindling model (Turski et al., 1983). Recently, organotypic hippocampal slice culture was developed as an *in vitro* TLE model by the Bausch and McNamara labs (Bausch and McNamara, 2004; Bausch et al., 2006).

Organotypic brain slice cultures were developed nearly 30 years ago. Currently, two techniques of brain slice cultures are frequently used for scientific research: the roller-tube and membrane interface methods. In roller-tube cultures developed by Gahwiler (Gahwiler, 1981), brain slices are embedded in a plasma clot or a collagen matrix on glass coverslips and then placed in flat-sided culture tubes containing a small amount of medium. Oxygen is provided for slice cultures through slow rotation of tubes to frequently change air-medium interface. The membrane interface cultures popularized by Stoppini et al. (1991) is characterized by brain slices placed on the top of a semiporous membrane at the interface of oxygen above and medium below. Slice cultures in the

roller-tube thin down to 50 µm from initial 400 µm preparations after a few weeks *in vitro*, which is 1/2 the thickness of the interface cultures (Gahwiler et al., 1997). This makes the interface cultures better sustain the three-dimensional cytoarchitecture of the original brain tissues. This advantage, coupled with its easy preparation and maintenance and easy drug treatment, makes the interface slice culture technique more widely used for electrophysiological experiments.

In the past, tissues from different brain regions including hippocampus (Routbort et al., 1999), the thalamus (Molnar and Blakemore, 1999), cerebral cortex (Letinic et al., 2002), cerebellum (Ghoumari et al., 2003), hypothalamus (Shinohara and Inouye, 1995), and brain stem structures (Jones et al., 1995) were cultured using the interface method. Hippocampal slice cultures are used as a model to investigate mechanisms underlying epileptogenesis (Bausch and McNamara, 2004; Bausch et al., 2006), cell death (Runden et al., 1998), synaptic plasticity (Bahr, 1995), and neurogenesis (Laskowski et al., 2005). Hippocampal slice cultures share many similarities with *in vivo* models of TLE: mossy fiber sprouting, synaptic reorganization and a latent period followed by subsequent expression of seizures (Bausch and McNamara, 2000, 2004; Bausch et al., 2006). Moreover, hippocampal slice cultures retain similar cytoarchitecture with all cell types and morphological characteristics of glia cells to *in vivo* animals (Gahwiler et al., 1997). Compared to *in vivo* animal models, hippocampal slice cultures have many advantages: ease of preparation and manipulation, ease of treatment with drugs and ease of accessibility for electrophysiological recordings (Bausch, 2008). In addition, neuronal circuitry in hippocampal slice cultures is more intact after a few weeks of cultures than that in acute hippocampal slices, in which neuronal axons and dendrites are partially

severed during slice preparation (Bausch, 2008). This is because the remaining axons and dendrites severed during culture preparation regrow and form synaptic contacts with existing and newly growing axons and dendrites *in vitro* (Bausch, 2008).

Likewise, compared to *in vivo* animal models, hippocampal slice cultures show significant disadvantages that include loss of afferent pathways into hippocampus, an altered physiological milieu and the absence of seizure-related behaviors (Bausch, 2008). On the other hand, deafferentation due to removal of entorhinal cortex might induce aberrant axonal sprouting and synaptic reorganization, similar to the etiology of epilepsy (Bausch, 2008). However, there is no perfect model of temporal lobe epilepsy, and hippocampal slice cultures are still a promising model to begin to investigate potential mechanisms underlying differential effects of NMDAR antagonists on epileptogenesis.

Effects of distinct classes of NMDAR antagonists on seizure expression in hippocampal slice cultures

The long-term goal of the Bausch's lab is to understand the roles of NMDAR in normal brain function and in different classes of NMDAR antagonists in the prevention of epileptogenesis. Our lab previously investigated effects of chronic treatment with different classes of NMDAR antagonists on electrographic seizures involving dentate granule cells from hippocampal slice cultures. Electrographic seizures induced by a $GABA_AR$ antagonist (BMI, 10 μM) or removal of Mg^{2+} (0 mM) were dramatically reduced in total seizure duration and number following chronic treatment with Ro25,6981 (1 μM), but were significantly increased following chronic treatment with D-APV (50

μM) (Fig 4). However, effect of chronic treatment with NVP-AAM077 (50 nM) on seizure expression remains under investigation.

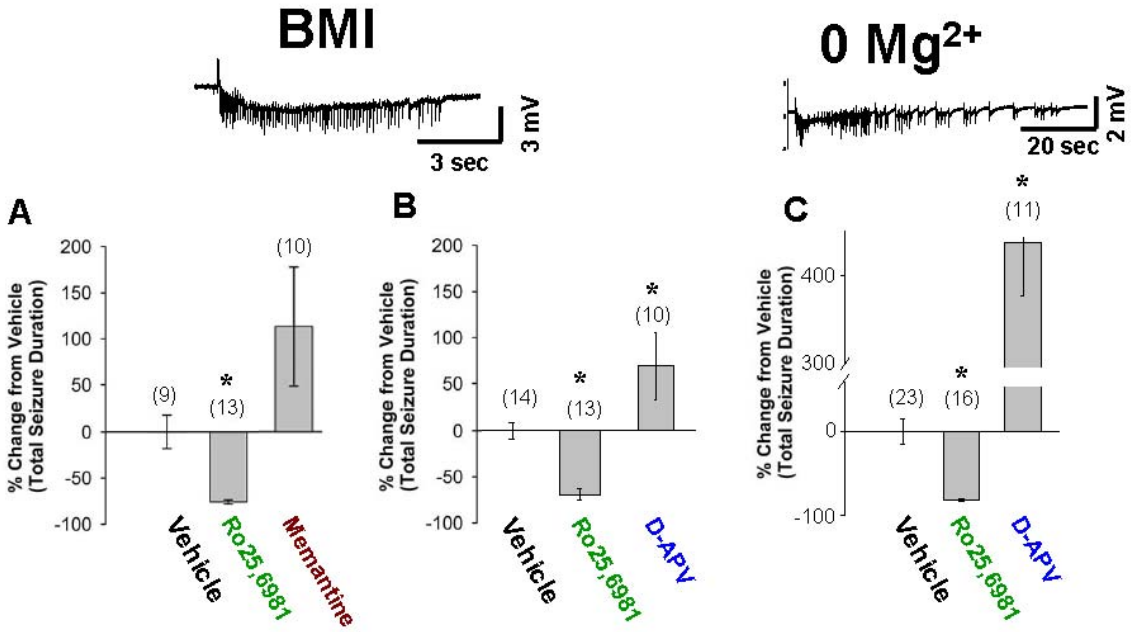


Figure 4A, adapted from Wang and Bausch, 2004;
Figure 4B and 4C, adapted from Society of Neurosci. Abstract, Dong and Bausch, 2005

Figure 4. Electrographic seizures induced by acute blockade of $GABA_A$ receptors or acute removal of Mg^{2+} block for NMDARs were dramatically reduced following chronic inhibition of NR2B-containing NMDAR with Ro25,6981, whereas were significantly increased following chronic NMDAR blockade with D-APV. Extracellular field potentials were recorded in physiological recording buffer containing BMI (10 μM) to block $GABA_A$ receptors or 0 mM Mg^{2+} recording buffer to unblock NMDARs in the dentate granule cell layer of hippocampal slice cultures treated with different classes of NMDAR antagonists. ***Tops*** show a representative trace for BMI-induced seizures for ***bottom A*** and ***B***, and a representative trace for 0 mM Mg^{2+}-induced seizures for ***bottom C***. Data are plotted as percentage change from vehicle. Bars indicate mean ± SEM. Numbers of cultures are indicated in parentheses. *, $p < 0.05$; different than vehicle; ANOVA by Ranks with Dunn's post hoc comparison.

The goal of my thesis project was to investigate the potential mechanisms underlying the differential effects of chronic treatment with different classes of NMDAR antagonists on seizure expression. Understanding the role of NMDAR activation in epileptogenesis would provide a rational basis for the development of novel antiepileptic agents. Since a reduction in excitatory networks and an increase in inhibitory networks can shift the balance between excitation and inhibition toward a decrease in neuronal network excitability,

I hypothesized that: 1) chronic treatment with D-APV or memantine will increase and chronic treatment with Ro25,6981 will reduce connectivity and synaptic transmission in excitatory networks; 2) chronic treatment with Ro25,6981 will increase connectivity and synaptic transmission in inhibitory networks onto individual granule cells; and 3) these changes will correlate with BMI- or 0 mM Mg^{2+}-induced seizure expression.

To test this hypothesis, in all of my studies hippocampal slice cultures were chronically treated for the entire 17-21 day culture period with vehicle or different classes of NMDAR antagonists [Ro25,6981 (1 µM), NR2B subunit-selective; NVP-AAM077 (50 nM), NR2A subunit-selective; memantine (1 µM), moderate-affinity uncompetitive; D-APV (50 µM), high-affinity competitive]. After wash out of NMDAR antagonist for ≥20 mins, whole cell electrophysiological recording were used to examine electrical membrane properties and synaptic changes in the dentate gyrus. Single neurons were filled with neurobiotin, followed by digital reconstructions and analyses to assess cell morphology.

Aim 1: To determine if glutamatergic transmission onto granule cells was suppressed following chronic treatment with Ro25,6981, but was enhanced following chronic treatment with D-APV, memantine or NVP-AAM077.

BMI-induced seizures were reduced following chronic treatment with Ro25,6981, but increased following chronic treatment with D-APV. Since GABAergic transmission was blocked while recording BMI-induced seizures, changes in glutamatergic transmission and electrical properties could directly contribute to alterations in BMI-induced seizures. To investigate this idea, we studied changes in: (1) granule cell electrical membrane properties; (2) granule cell morphology; as well as (3) spontaneous and miniature excitatory postsynaptic currents (Fig 5); (4) NMDAR-mediated miniature EPSCs ($mEPSCs_{NMDAR}$) and (5) tonic NMDAR currents in granule cells.

Aim 2: To determine if GABAergic synaptic transmission onto individual granule cells was enhanced following chronic treatment with Ro25,6981 and investigate potential mechanisms underlying these changes in GABAergic transmission.

0 mM Mg^{2+}-induced (Mg^{2+} free) seizures were reduced following chronic treatment with Ro25,6981, but increased following chronic treatment with D-APV. Since GABAergic transmission was intact while recording 0 mM Mg^{2+}-induced seizures, changes in GABAergic transmission and electrical properties could directly contribute to alterations in 0 mM Mg^{2+}-induced seizures. To investigate this hypothesis, we studied changes in sIPSCs and mIPSCs in dentate granule cells and investigated possible causes occurring in D/H border interneurons including: (1) inhibitory synaptic inputs, (2) electrical membrane and action potential properties, (3) action potential conduction failure, (4)

vesicle release probability and (5) the involvement of voltage-gated potassium channels in changes in action potential dependent GABAergic transmission.

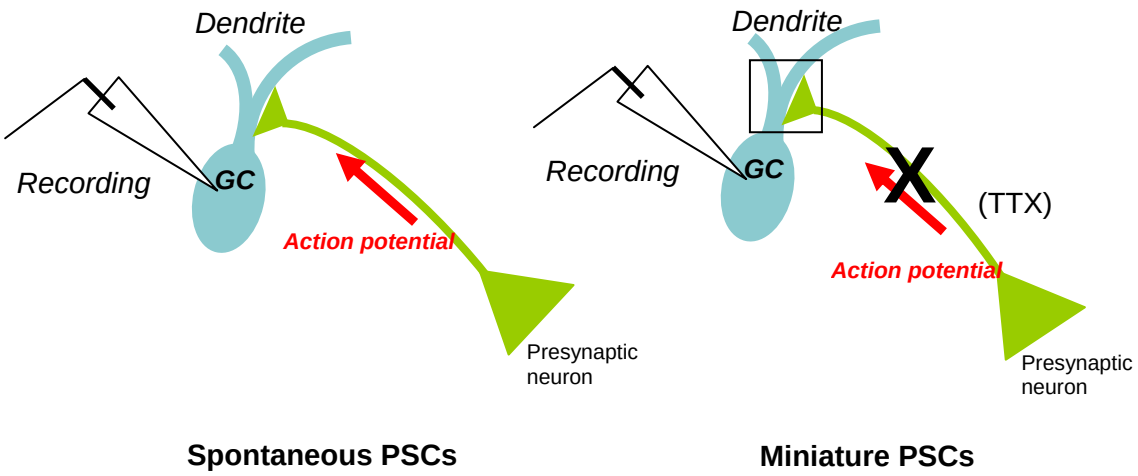


Figure 5. Introduction to spontaneous and miniature postsynaptic currents (PSCs). Spontaneous PSCs are action potential-dependent synaptic activities. Miniature PSCs are action potential-independent synaptic transmission and recorded in the presence of tetrodotoxin (TTX) to block action potentials. Miniature PSCs reflect synaptic activity in individual synapses (box). Changes in miniature PSC amplitude and charge transfer often point to postsynaptic mechanisms while an alteration in miniature PSC frequency usually indicates a presynaptic mechanism. A change in spontaneous but not miniature PSCs suggests an alteration in action potential-dependent neurotransmitter release.

Initial results not presented in subsequent Chapters

The initial results are presented here to better lead my subsequent studies in Chapter 2 and 3.

Large amplitude sEPSCs were altered differentially by chronic treatment with NMDAR antagonists.

To begin to assess changes in excitatory neurotransmission following chronic treatment with NMDAR antagonists, we first recorded spontaneous EPSCs in individual dentate granule cells. As described previously (Bausch and McNamara, 2004), sEPSCs in granule cells from vehicle-treated organotypic hippocampal slice cultures fell into two distinct amplitude distributions, <300 pA ($sEPSCs_{small}$; range 8.5-177 pA) and >2 nA ($sEPSCs_{large}$; range, 2.1-7.6 nA). Spontaneous $EPSCs_{large}$ were characterized by low frequency (0.026 ± 0.003 Hz), long duration (1874 ± 70 ms) and multiple large peaks (7.6 ± 0.4) (Fig. 6A) and mirrored temporally-associated epileptiform bursts recorded simultaneously with field potential recordings (Fig. 6B). Spontaneous $EPSCs_{large}$ were abolished by TTX or by a combination of D-APV and CNQX, but were only partially inhibited by either D-APV or CNQX alone (data not shown). These data suggest that $sEPSCs_{large}$ were action potential-dependent transmission in a recurrent polysynaptic circuit containing both NMDAR and AMPAR/KAR. Comparing the effects of chronic treatment with distinct classes of NMDAR antagonists, inhibition of NR2B-containing NMDAR with Ro25,6981 reduced $sEPSC_{large}$ frequency by 50% compared to vehicle and all other treatment groups, but had no significant effect on amplitude or duration (Fig. 7). Spontaneous $EPSC_{large}$ in cultures treated with the other NMDAR antagonists were similar to vehicle (Fig. 7). These findings are consistent with our hypothesis and previous

report describing opposite effects on pathological hyperactivity in excitatory networks following chronic NMDAR blockade with D-APV and inhibition of NR2B-containing receptors with Ro25,6981 (Wang and Bausch, 2004; Bausch et al., 2006).

In contrast to $sEPSC_{large}$, $sEPSC_{small}$ in granule cells from vehicle-treated cultures occurred were characterized by higher frequency (1.12 ± 0.17 Hz), shorter decay (7.3 ± 0.3 ms) and a single peak (Fig. 8). CNQX but not D-APV abolished spontaneous $EPSCs_{small}$ (data not shown), suggesting a predominant AMPAR/KAR component. Consistent with our hypothesis, chronic NMDAR blockade with D-APV increased $sEPSC_{small}$ frequency, and caused a small but significant increase in amplitude and charge transfer compared to vehicle (Fig.8). However, contrary to our hypothesis, chronic inhibition of NR2B-containing NMDAR with Ro25,6981 or NR2A-containing NMDAR with NVPAAM077 caused a small but significant increase in $sEPSC_{small}$ frequency, amplitude and charge transfer. Spontaneous $EPSCs_{small}$ in memantine-treated cultures treated were similar to vehicle (Fig. 8).

Figure 6. Representative traces of sEPSCs

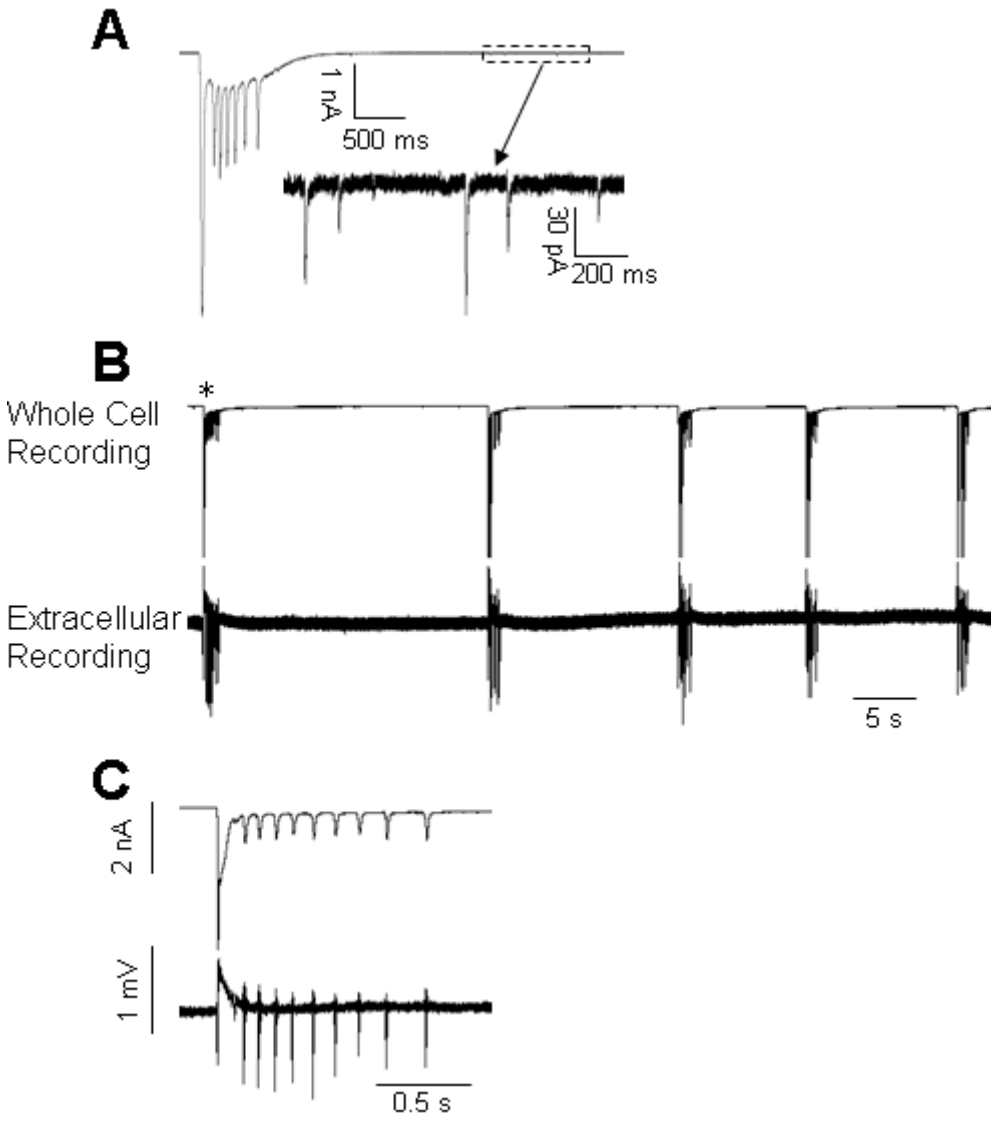


Figure 6. Representative traces of spontaneous EPSCs. Traces were obtained from a vehicle-treated culture and recorded in physiological buffer containing BMI (10 μM). Recording of sEPSCs was conducted at a -70 mV holding potential in physiological recording buffer containing BMI (10 μM) and pipette solution containing QX-314 (5 mM) to block action potentials. ***A,*** traces illustrate ***Top,*** a large amplitude sEPSC ($sEPSC_{large}$) and ***Bottom,*** small amplitude sEPSCs ($sEPSC_{small}$) expanded from the boxed region of the top trace. For quantitative data, see Fig. 7 for $sEPSC_{large}$ and Fig. 8 for $sEPSC_{small}$. Paired whole-cell and field potential recordings revealed that $sEPSCs_{large}$ mirrored epileptiform bursts. ***B, top*** $sEPSCs_{large}$ were recorded from a single granule cell using whole-cell recording at a holding potential of -70 mV. ***Bottom*** field potentials were recorded in the dentate granule cell layer using extracellular recording. ***C,*** shows an expanded time scale of the large amplitude sEPSC/epileptiform event indicated by * in panel B. Vertical scale bars in C also apply to traces in B.

Figure 7. Large amplitude sEPSCs

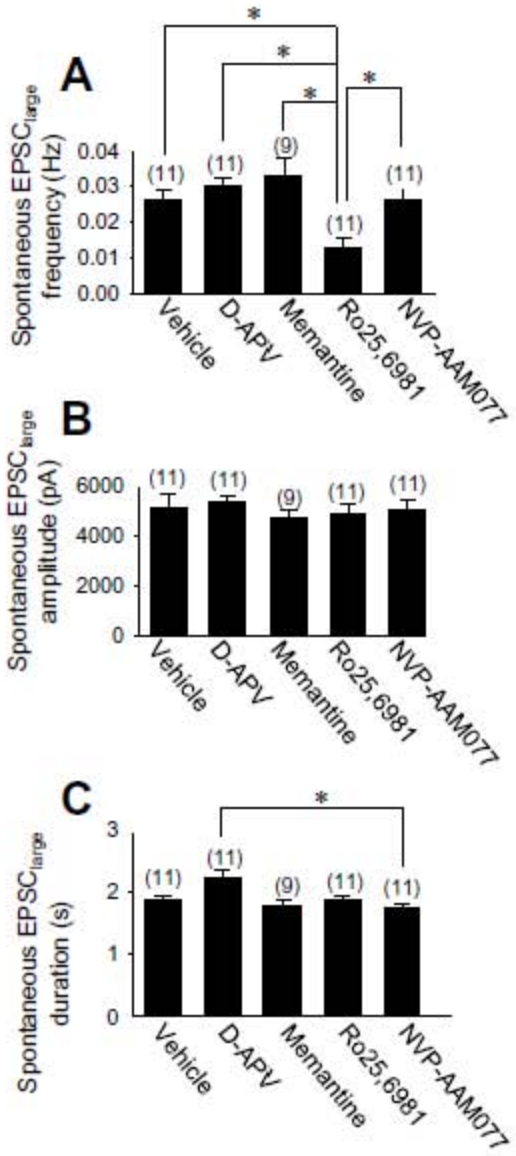


Figure 7. Large amplitude sEPSC frequency was significantly reduced following chronic treatment with Ro25,6981. ***A,*** bar graph revealed significantly reduced $sEPSC_{large}$ frequency following chronic treatment with Ro25,6981 compared to vehicle and other antagonist treatment groups, but no significant change following chronic treatment with other classes of NMDAR antagonists. ***B,*** $sEPSC_{large}$ amplitude was similar between different treatment groups. ***C,*** shows a significant increase in $sEPSC_{large}$ duration following chronic treatment with D-APV compared to chronic treatment with NVP-AAM077. *, $p<0.05$, ANOVA with Holm-Sidak *post hoc* comparison. The number of granule cells/slice cultures is indicated in parentheses.

Figure 8. Small amplitude sEPSCs

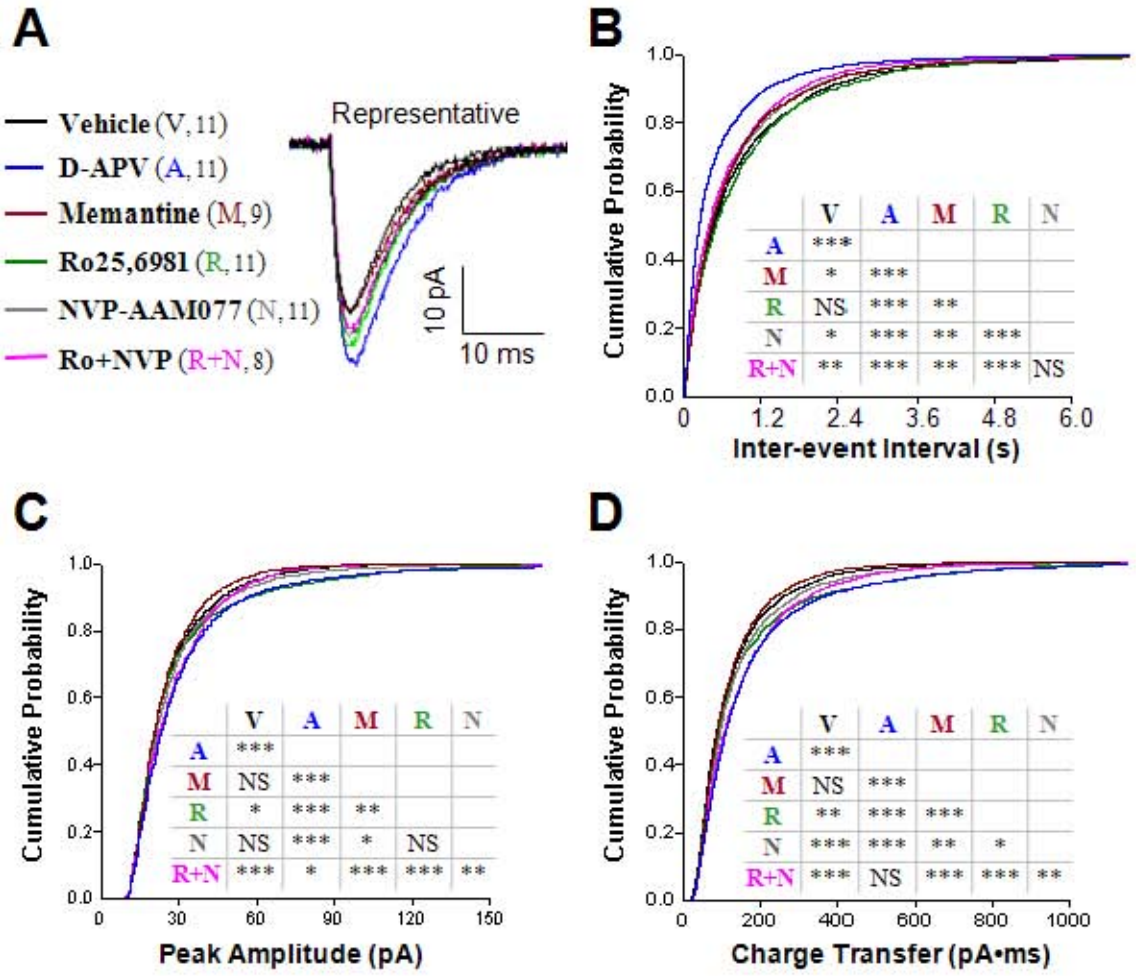


Figure 8. Small amplitude sEPSCs ($sEPSCs_{small}$) in granule cells were enhanced in D-APV-treated hippocampal slice cultures, but were only modestly altered in cultures treated with the other NMDAR antagonists. Recordings were conducted at a -70 mV holding potential in physiological recording buffer containing BMI (10 μM) and pipette solution containing QX-314 (5 mM) to block action potentials in the recorded neurons. ***A)*** Averages of all $sEPSC_{small}$ from representative granule cells illustrate increased amplitude for D-APV relative to vehicle and all other NMDAR antagonists. Cumulative probability plots revealed significant increases in $sEPSC_{small}$ ***B)*** frequency, ***C)*** peak amplitude, and ***D)*** charge transfer in granule cells from slice cultures treated with D-APV that were more robust than in cultures treated with the other NMDAR antagonists. ***Insets,*** tables show statistical comparisons between treatment groups. * $p \leq 0.025$; ** $p \leq 0.005$; *** $p \leq 0.0000$; NS, not significant; two-tailed Kolmogorov-Smirnov test. Legend in A applies to A-F; the number of granule cells/slice cultures is indicated in parentheses.

Action potential threshold was slightly more negative following chronic NMDAR blockade and inhibition of NR2A-containing NMDAR.

Alterations in action potential and membrane properties could contribute to changes in sEPSCs and therefore were examined using whole-cell current-clamp recordings. Comparison between treatment groups revealed no differences in granule cell: 1) resting membrane potential; 2) input resistance; or 3) action potential number at threshold or following a 200 pA input (Table 1). Action potential threshold in Ro25,6981- and memantine-treated cultures was similar to vehicle. These data suggest that alterations in action potential and membrane properties did not underlie changes in sEPSCs following chronic inhibition of NR2B-containing NMDAR with Ro25,6981. Action potential threshold however was slightly, but significantly more negative (-1.5 mV) in granule cells from cultures treated with D-APV, NVP-AAM077 and the combination of Ro25,6981 and NVP-AAM077 when compared to cultures treated with vehicle (Table 1). These findings implicate NR2A as a modulator of sodium channel function. While this small shift in action potential threshold is unlikely to elicit dramatic hyperexcitability by itself, it could act in concert with other pro-excitatory changes in D-APV- and NVPAAM077-treated cultures. Hence, we next investigated changes in excitatory and inhibitory circuits onto individual dentate granule cells because these changes would shift the balance between excitation and inhibition in granule cells and alter neuronal excitability.

Table 1. Granule cell membrane properties

Treatment	n	RMP (mV)	R_{IN} (MΩ)	AP Threshold (mV)	AP # at Threshold	AP # at 200 pA
Vehicle	50	-58.5 ± 0.2	149 ± 7	-36.4 ± 0.3	2.0	6.0
D-APV	41	-59.3 ± 0.3	136 ± 7	-37.9 ± 0.3[a, c]	2.0	6.0
Memantine	58	-59.7 ± 0.3	133 ± 5	-36.4 ± 0.2	2.0	5.0
Ro25,6981	53	-59.0 ± 0.3	148 ± 6	-36.9 ± 0.3	2.0	6.0
NVP-AAM077	59	-59.1 ± 0.3	136 ± 5	-37.4 ± 0.2[a]	2.0	6.0
Ro+NVP	60	-59.1 ± 0.3	148 ± 5	-37.4 ± 0.2[a]	2.0	6.0

Means ± SEM (RMP, R_{IN}, AP Threshold); medians (AP #). *Abbreviations*: RMP, resting membrane potential; R_{IN}, input resistance; AP threshold, action potential threshold; AP # at threshold, the number of AP elicited at threshold; AP # at 200 pA, the number of AP following a 200 pA current. n, the number of granule cells / hippocampal slice cultures investigated. [a], different than vehicle; [c], different than memantine; $p<0.05$, ANOVA with Holm-Sidak *post hoc* comparison.

Chapter 2

Chronic subunit selective NMDA receptor inhibition induces plasticity in excitatory hippocampal formation circuits.

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Abstract

Chronic NMDAR blockade with high-affinity competitive and uncompetitive antagonists leads to homeostatic changes in synaptic connectivity and glutamate receptor expression. The long-term impact of more clinically relevant classes of NMDAR antagonists or subunit-selective antagonists remains to be determined. The purpose of this study was to compare the consequences of chronic NMDAR inhibition with the high-affinity competitive antagonist D-APV, the moderate-affinity uncompetitive NMDAR antagonist memantine, the allosteric NR2B-selective NMDAR antagonist, Ro25,6981 and the newer competitive NR2A-prefering NMDAR antagonist, NVP-AAM077 on excitatory networks involving granule cells from organotypic hippocampal slice cultures.

Electrophysiological recordings revealed increased mEPSC amplitude and charge transfer following chronic treatment with all NMDAR antagonists, consistent with AMPAR up-regulation. The magnitude of these increases correlated with the degree of NMDAR inhibition. Increased mEPSC frequency following chronic treatment with all NMDAR antagonists except NVP-AAM077 suggested increased synapse number. Indeed, the number of excitatory synapses onto granule cell dendrites measured immunohistochemically was increased following chronic treatment with all NMDAR antagonists except Ro25,6981. Dramatic reductions in NMDAR-mediated mEPSC amplitude and frequency following chronic D-APV and large increases in tonic astrocyte-mediated NMDAR currents following chronic Ro25,6981 also were observed. These changes may contribute to the therapeutic efficacy of NMDAR antagonists and / or represent homeostatic changes with limited effects on disease processes.

Introduction

N-methyl-D-aspartate receptors (NMDAR) are heteromeric ligand-gated ion channels composed of NR1 and NR2 (A-D) subunits, with NR2A and NR2B being the most prominent subunits in hippocampus and cortex (Monyer et al., 1994; Yamakura and Shimoji, 1999). NMDAR activation is required for normal brain function including synaptic plasticity, learning and memory and neuronal survival (Morris et al., 1986; Mori and Mishina, 1995; Dingledine et al., 1999; Hardingham and Bading, 2003). Excessive NMDAR activation can contribute to pathophysiology associated with epilepsy, brain trauma, stroke, depression, and neurodegenerative disorders, which led to the development of NMDAR antagonists for therapeutic use (Dingledine et al., 1990; Lipton, 2007).

Early high-affinity competitive and uncompetitive antagonists that dramatically reduce global NMDAR function caused cognitive and psychotomimetic dysfunction. Chronic use of these antagonists lead to neuronal vacuolization, paradoxical seizure exacerbation and increased synaptic connectivity and ionotropic glutamate receptor expression (Olney et al., 1989; Sveinbjornsdottir et al., 1993; Gould et al., 1994; Muir and Lees, 1995; Loscher, 1998; Ikonomidou et al., 1999; Parsons et al., 1999; Wang and Bausch, 2004). A number of newer NMDAR antagonists appear to reduce pathological over-activation while better maintaining the physiological NMDAR function required for normal brain function. Memantine is a moderate-affinity uncompetitive NMDAR antagonist currently used clinically to treat Parkinson's disease, dementia, and spasticity (Parsons et al., 1999). The newer allosteric NR2B-selective NMDAR antagonists remain in clinical trials, but are well-tolerated in broad patient populations (Muir and Lees, 1995;

Palmer, 2001; Kemp and McKernan, 2002). However, despite the obvious clinical relevance the physiological consequences of chronic treatment with these NMDAR antagonists at the cellular and network levels are largely unknown. This information is necessary to understand the impact of NMDAR activation on the physiological properties of neuronal circuits and how brain networks adapt to changes in NMDAR function.

The purpose of this study was to compare the consequences of chronic treatment with the moderate-affinity uncompetitive NMDAR antagonist memantine, the allosteric NR2B-selective NMDAR antagonist, Ro25,6981 and the newer competitive NR2A-prefering NMDAR antagonist, NVP-AAM077 on excitatory networks. We focused on excitatory networks because of the documented effects of chronic NMDAR blockade on excitatory neurotransmission (Rao and Craig, 1997; Liao et al., 1999). Organotypic hippocampal slice cultures were used because they maintain intrinsic hippocampal networks and layer-specific projections (see Bausch 2009). We hypothesized that chronic treatment with D-APV or memantine would promote plasticity that increased, while Ro25,6981 would support plasticity that decreased excitatory transmission. This hypothesis was based upon our initial studies showing that electrographic seizures were exacerbated following chronic treatment of organotypic hippocampal slice cultures with D-APV or memantine, but were dramatically reduced following chronic treatment with Ro25,6981 (Wang and Bausch, 2004).

Materials and Methods

Choice of antagonists. Representative NMDAR antagonists were chosen based on their pharmacological properties as described previously (Wang and Bausch, 2004). Concentrations were selected to: 1) elicit maximal antagonism while maintaining selectivity; 2) allow direct comparison with our previous studies (Wang and Bausch, 2004; Bausch et al., 2006); and 3) (except for D-APV) elicit roughly similar degrees of NMDAR inhibition.

D-APV (50 μM) was chosen as the high-affinity competitive NMDAR antagonist because of its high affinity and selectivity for the NMDAR (Evans et al., 1982) and its frequent use in physiological experiments. D-APV (50 μM) completely blocks NMDAR-mediated responses (IC_{100}) and was included to facilitate comparison with previous studies documenting neuroplasticity following NMDAR blockade.

Memantine (1 μM) was chosen as the moderate-affinity uncompetitive NMDAR antagonist because it is used clinically and is reasonably selective for NMDAR at concentrations up to 1 μM (Parsons et al., 1999), although nonspecific effects at some subtypes of serotonin (5-HT3) and nicotinic acetylcholine (α9-α10 heteromers and α7 homomers) receptors have been reported (Johnson and Kotermanski, 2006). Memantine (1 μM) causes partial blockade (~IC_{50} at -100 to -70 mV; see Parsons et al., 1999) and slightly less than 1 μM memantine is found in serum and cerebrospinal fluid following administration of clinically tolerated doses of memantine (Kornhuber and Quack, 1995).

Ro25,6981 (1 μM) was selected as the NR2B-selective antagonist because Ro25,6981 has high affinity and specificity for NR2B-containing NMDAR (Fischer et al., 1997). Ro25,6981 was chosen over ifenprodil because ifenprodil also interacts with β-

adrenergic receptors, serotonin receptors and calcium channels (Chenard et al., 1991; Church et al., 1994; McCool and Lovinger, 1995). Ro25,6981 (1 μM) imparts maximal binding at NR2B-containing NMDAR, but the degree of functional NMDAR inhibition is use-dependent and thus is proportional to the level of NMDAR activation. The greatest inhibition (~80%) occurs at very high glutamate concentrations, while a slight potentiation occurs at very low glutamate concentrations since ifenprodil derivatives, such as Ro25,6981 increase NMDAR affinity for glutamate (Kew et al., 1996; Fischer et al., 1997).

NVP-AAM077 (50 nM) was chosen as the NR2A-prefering antagonist because of its modest selectivity for NR2A-containing NMDAR at this concentration. NVP-AAM077 (50 nM) causes partial blockade (~IC_{60}) (Auberson et al., 2002; Feng et al., 2004; Neyton and Paoletti, 2006).

Organotypic hippocampal slice cultures. Slice cultures were prepared using the method of Stoppini (Stoppini et al., 1991) as described previously (Bausch and McNamara, 2000; Bausch et al., 2006). All treatment of animals was according to National Institutes of Health, Department of Defense and institutional guidelines. Briefly, postnatal day 10-11 Sprague-Dawley rat pups (Taconic, Germantown, NY) were anesthetized with pentobarbital and decapitated. Brains were removed. Hippocampi were cut into 400 μm transverse sections using a McIlwain tissue chopper and placed into Gey's balanced salt solution (GBSS) composed of (in mM): 137 NaCl, 5 KCl, 0.25 $MgSO_4$, 1.5 $CaCl_2$, 1.05 $MgCl_2$, 0.84 Na_2HPO_4, 0.22 K_2HPO_4, 2.7 $NaHCO_3$, 41.6 glucose. The entorhinal cortex was removed and the middle 4-6 slices of each hippocampus were placed onto tissue culture membrane inserts (Millipore, Bedford, MA) in a culture dish

containing medium. Medium consisted of 50% minimum essential medium, 25% Hank's buffered salt solution, 25% heat-inactivated horse serum, 0.5% GlutaMax, 10 mM HEPES (all from Invitrogen, Carlsbad, CA) and 6.5 mg/ml glucose (pH 7.2). Slice cultures were maintained at 37°C under room air + 5% CO_2 and medium was changed three times per week. Slice cultures were treated within 10-15 min. of preparation-induced injury and deafferentation [i.e. the time of plating (0 days *in vitro* (DIV)] through the end of the culture period (17-21 DIV) with D(-)-2-amino-5-phosphonopentanoic acid (D-APV, 50 µM; Tocris Cookson, Ellisville, MO), memantine hydrochloride (1 µM; Tocris Cookson), Ro25,6981 hydrochloride (1 µM; Sigma), NVP-AAM077 (50 nM; kind gift from Dr. Yves Auberson, Novartis Institutes for Biomedical Research, Basel, Switzerland), or Ro25,6981 (1 µM) + NVP-AAM077 (50 nM) (Ro+NVP), diluted in medium. For vehicle-treated slice cultures, drugs were omitted. Cultures treated with vehicle and NMDAR antagonists were always studied concurrently under identical experimental conditions. Only cultures that displayed bright, well-defined cell layers were used.

Electrophysiological recordings. Recordings were conducted in dentate granule cells from the suprapyramidal blade of the granule cell layer following >20 min. washout of treatment drugs as described previously (Bausch et al., 2006). Our previous results showed differential effects of chronic treatment with different classes of NMDAR antagonists on seizures involving granule cells (Wang and Bausch, 2004) and the dentate granule cells are thought to act as a "gate" to invasion of pathological hyperexcitability into the hippocampus (Collins et al., 1983; Behr et al., 1996; Behr et al., 1998). Briefly, a single cultured slice attached to the tissue culture insert membrane was placed into a

submerged recording chamber mounted to a Zeiss Axioskop microscope with IR-DIC optics (Carl Zeiss Inc., Thornwood, NY). Slice cultures were superfused (2-3 ml/min) at room temperature with physiological recording buffer composed of (in mM): 124 NaCl, 4.9 KCl, 1.2 KH_2PO_4 2.4 $MgSO_4$, 2.5 $CaCl_2$, 25.6 $NaHCO_3$, and 10 glucose or nominally 0 mM Mg^{2+} buffer containing 124 NaCl, 4.9 KCl, 1.2 KH_2PO_4 2.4 Na_2SO_4, 2.5 $CaCl_2$, 25.6 $NaHCO_3$, and 10 glucose, equilibrated with 95% O_2, 5% CO_2. Tetrodotoxin (TTX, 1 µM; Sigma), D-APV (50 µM), bicuculline methiodide (BMI, 10 µM; Tocris Cookson), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM; Tocris Cookson), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline -7-sulfonamide disodium salt (NBQX, 10 µM; Tocris Cookson), D,L-threo-β-benzyloxyaspartate (DL-TBOA, 50 µM; Tocris Cookson), Ro25,6981 (1 µM), NVP-AAM077 (500 nM), [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid monoester (MRS2365, 3 µM; Tocris Cookson) and (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]- 2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester (MRS2500, 1 µM; Tocris Cookson) were diluted immediately prior to use and acutely applied by bath superfusion. Recording pipettes were filled with (in mM): K-gluconate 125, KCl 13, HEPES 10, EGTA 10, MgATP 2 (pH 7.2 with KOH) for all whole-cell recordings except those measuring NMDAR-mediated currents. For miniature and tonic NMDAR-mediated currents, recording pipettes were filled with (in mM): Cs-methanesulfonic acid 125, CsCl 13, HEPES 10, EGTA 10, Na_2ATP 2 (pH 7.2 with CsOH). Recordings were excluded if the RMP was more positive than -50 mV or series resistance varied more than 15%. Data were collected using a Multiclamp 700A amplifier (2 kHz analog filter), Digidata 1320 A/D

converter, and sampled at 10 kHz using pCLAMP software (Axon Instruments, Union City, CA).

Electrophysiological analyses. MiniAnalysis software (Synaptosoft Inc., Fort Lee, NJ) was used for analyses of all postsynaptic currents and generation of cumulative probability plots. Detection threshold was set at 8 pA, rise and decay times were measured from 0-100% and 100-10%, respectively.

NMDAR-mediated slow inward currents (SICs) were fit with the product of exponential functions (best fit based on correlation coefficients; (Fellin et al., 2004) and analyzed using Clampfit software (Axon Instruments). Amplitude was measured at peak negativity, rise time was measured at 10-90% and decay time constant was calculated from the exponential fit. SICs were defined as events with >40 ms rise time (Angulo et al., 2004; Fellin et al., 2004; Fiacco et al., 2007).

Tonic NMDAR currents were high-pass filtered at 1 Hz (8-pole Bessel, 3 dB cutoff) and low-pass filtered at 1 kHz (8-pole Butterworth, 3 dB cutoff) using Clampfit software. Power spectra (0.61 Hz resolution) were generated from 1 min. data segments immediately prior to application of: 1) 0 mM Mg^{2+} recording buffer; 2) D,L-TBOA; 3) NVP-AAM077; 4) physiological recording buffer; and 5) D-APV as well as 6) at the peak of the TBOA-induced tonic current. Using the Levenberg-Marquardt algorithm, the TBOA-induced spectra was fit with the sum of two Lorentzian functions: $S(f)=S(0)_1/[1+(f/f_{c1})^2] + S(0)_2/[1+(f/f_{c2})^2]$, where S(f) is the power at a given frequency; $S(0)_1$ and $S(0)_2$ are the powers for component 1 and 2 at zero frequency, respectively; f_{c1} and f_{c2} are the corner frequencies at which the spectral power is 1/2 $S(0)_1$ and 1/2 $S(0)_2$,

respectively. The mean open time (τ) for each component was calculated as $\tau = 1/(2\pi f_c)$ (Sah et al., 1989; Kohr et al., 1993).

Single channel conductance was estimated using stationary mean-variance noise analysis. The mean noise and variance from the mean were calculated from a 200 ms data segment during the slow rise phase of the TBOA-induced tonic NMDAR-mediated current. Single channel currents (I) were estimated as the slope of the linear portion of the mean-variance plot. Single channel conductance (g) was calculated as g = I/V, where V is the -70 mV holding potential. Channel number was calculated as tonic current amplitude / single channel current (Kohr et al., 1993; Tammaro and Ashcroft, 2007).

Neurobiotin labeling and morphometric analyses. Individual granule cells were filled with neurobiotin (0.4-0.5% in the pipette solution; Vector, Burlingame, CA) during whole-cell recordings and visualized as described previously (Bausch et al., 2006). Briefly, after electrophysiological experiments, cultures were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), removed from the insert membrane, sunk in 30% sucrose in 0.1 M PBS [PB containing 0.15 M NaCl and 2.7 mM KCl (pH 7.4)] and stored at –70^{o}C. Thawed cultures were treated with 10% methanol and 0.6% H_2O_2, in PBS, blocked with 2% bovine serum albumin (BSA) and 0.75% Triton X-100 in PBS and incubated overnight at 4^{o}C in ABC elite (Vector) diluted according to kit instructions in PBS containing 2% BSA and 0.1% Triton X-100. Cultures were then treated with 0.05% 3,3'-diaminobenzidine (DAB, Sigma), 0.028% $CoCl_2$, 0.02% $NiSO_4{\cdot}(NH_4)SO_4$ and 0.00075% H_2O_2 in PBS until staining was apparent. Cultures were mounted onto subbed glass slides, dehydrated, cleared and coverslipped.

Three-dimensional digital reconstructions were drawn manually and analyzed using Neurolucida software (MicroBrightField Inc., Colchester, Vermont), a Zeiss Axioskop microscope equipped with 63X oil objective, MicroFire CCD camera (Optronics Inc., Goleta, CA), and motorized stage and focus encoder (Ludl Electronic Products Ltd., Exton, PA) as described previously (Bausch et al., 2006). Regions were defined as: molecular layer, supragranular regions of the dentate gyrus; granule cell layer, tightly packed layer of granule cells; hilus, region between the blades of the granule cell layer excluding the CA3c pyramidal cell layer; and CA3, CA3a-c pyramidal cell layers and proximal dendrites in CA3a/b. Branch points were defined as points of bifurcation. Ends were defined as points of termination. Putative boutons were defined as enlargements of $\geq$2 times the width of the adjacent axon.

Immunohistochemistry. Slice cultures were fixed with 4% paraformaldehyde in PBS for 20 min, removed from the membrane and processed for immunohistochemistry as described previously (Bausch et al., 2006). All steps were performed at RT unless stated otherwise. Briefly, free floating slice cultures were pretreated sequentially with 70% ethanol, 100% methanol, 70% ethanol, and 7% streptavidin in PBS followed by 7% biotin in PBS. Cultures were blocked for 1 hr. at 37°C with PBS containing 2% gelatin and 10% normal goat serum. Slice cultures were then processed for double immunofluorescence against microtubule associated protein (MAP2) to label dendrites and vesicular glutamate transporter 1 (vGlut1) to label glutamatergic terminals. Slice cultures were incubated for 1 hr. at RT followed by 36 hr at 4°C with mouse monoclonal anti-MAP2 IgG (clone HM-2 ascites; Sigma) diluted 1:1,000 and guinea pig polyclonal anti-vGlut1 antibody (Chemicon) diluted 1:1000 in PBS containing 2% BSA, 10% normal goat serum, and

0.1% Triton X-100. Immunoreactivity was visualized with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) diluted 1:1,000 and Alexa 555-conjugated goat anti-guinea pig IgG (Molecular Probes) diluted 1:1,000 in PBS containing 1% BSA and 0.1% Triton X-100 for 1 hr. Slice cultures were then mounted onto subbed glass slides and coverslipped with Vectashield mounting media (Vector, Burlingame, CA).

Images were collected using a Zeiss Pascal LSM5 confocal microscope, 63x oil objective and multi-track scanning with an Argon laser and 405/488/543 nm excitation, 505- to 530-nm band-pass emission and 560-nm long-pass emission filters. Acquisition parameters were adjusted to minimize photobleaching and background labeling and were identical across treatment groups. Z-series were collected from 5 to 10 consecutive optical sections (2,048 x 2,048 pixels, 0.1 µm / pixel; 0.3 µm *z*-axis interval). Z-stack reconstructions were used to identify dendrites; granule cells were identified morphologically. Only dendrites clearly attributable to a single granule cell were used for further analyses. All analyses were performed on background-subtracted images. The number of vGlut1-immunoreactive (-IR) puncta apposed to a single granule cell dendrite was counted manually from individual optical sections. Puncta were counted only once, even if they appeared in more than one sequential image. The integrated intensity of vGlut1-IR puncta was measured following spectral separation to isolate vGlut1 immunoreactivity. Images were thresholded to define puncta area and the intensity of vGlut1 immunoreactivity was measured within the thresholded region. Data from 5 consecutive serial sections for each granule cell dendrite were averaged. All quantitative analyses were performed with Metamorph software (Molecular Devices; Downingtown, PA)

Statistics. Investigators were blinded to experimental groupings for all data analyses. Parametric data were represented as means $\pm$ SEM. Nonparametric data were represented as medians. Most statistical analyses were performed with Sigma Stat software (SPSS Inc., Chicago, Illinois). Data fitting a parametric distribution were tested for significance using an ANOVA with Holm-Sidak post hoc comparison (multiple groups) or t-test (two groups). Data fitting a non-parametric distribution were tested for significance using ANOVA on Ranks. Proportions were tested for significance using a Chi-squared test. Significance was defined as $p \leq 0.05$. Cumulative probability distributions were tested for significance using a two-tailed Kolmogorov-Smirnov test using MiniAnalysis software; significance was defined as $p \leq 0.025$.

Results

Increases in mEPSC amplitude correlated with degree of blockade, but alterations in frequency were dependant on NMDAR antagonist class.

We used whole-cell voltage clamp recordings of action potential-independent miniature EPSCs (mEPSCs) in dentate granule cells to document changes in excitatory circuitry and ionotropic glutamate receptor function. Miniature EPSCs were measured at a holding potential of -70 mV and were abolished by CNQX but not D-APV (not shown), suggesting a predominant AMPAR/KAR component altered miniature postsynaptic current (mPSC) amplitude and charge transfer often point to postsynaptic mechanisms while altered mPSC frequency usually indicates a presynaptic mechanism. Comparison between treatment groups revealed that chronic NMDAR blockade with D-APV dramatically increased mEPSC amplitude and charge transfer compared to vehicle. NMDAR inhibition with other antagonists caused intermediate increases in mEPSC amplitude and charge transfer (Table 1; Fig. 1C, D), suggesting that postsynaptic glutamate receptor function was increased in proportion to the degree of NMDAR inhibition. In addition to postsynaptic alterations, we also observed changes often ascribed to presynaptic mechanisms. Chronic NMDAR blockade with D-APV and inhibition of NR2B-containing NMDAR with Ro25,6981 increased mEPSC frequency compared to vehicle. These data suggest increased excitatory synapse number and/or glutamate release probability in cultures treated with D-APV or Ro25,6981.

Excitatory synapses onto granule cells were increased following NMDAR inhibition with all antagonists except Ro25,6981.

Chronic NMDAR blockade with high-affinity competitive antagonists, such as D-APV can promote synaptic reorganization and increase synapse number (Lin and Constantine-Paton, 1998; McKinney et al., 1999; Bausch et al., 2006). However, chronic NMDAR inhibition with other distinct classes of NMDAR antagonists may differentially regulate synaptic circuits (Wang and Bausch, 2004). We focused first on mossy fiber sprouting because sprouting of granule cell mossy fiber axons into the inner molecular layer leads to recurrent polysynaptic circuits between granule cells and such changes could contribute to our differential effects of NMDAR antagonists on mEPSC frequency. To test this idea, we filled individual granule cells with neurobiotin and performed digital reconstructions and morphometric analyses (Fig. 2, Table 2). In partial agreement with our prediction, mossy fiber collaterals in the molecular layer were increased by 100%, 200%, and 300% compared to vehicle in memantine-, NVP-AAM077- and D-APV-treated cultures respectively, but changes were not significant. No differences in the number of synaptic bouton-like swellings were noted (Table 2). Given our previously reported increases in supragranular Timm stained mossy fibers in organotypic hippocampal slice cultures treated chronically with D-APV or memantine (Wang and Bausch, 2004), these data suggest that mossy fiber sprouting into the molecular layer may occur only in a small subset of granule cells (Franck et al., 1995).

As a second more global measure of excitatory synapses, we used double-immunofluorescence labeling for vGlut1 to detect presynaptic glutamatergic terminals and MAP2 to identify dendrites. Quantitative analyses revealed that the intensity of

vGlut1 immunoreactivity in individual puncta apposed to granule cell dendrites was increased in cultures treated with D-APV or memantine when compared to vehicle (Fig. 3C), suggesting a possible up-regulation in vGlut1 transporter expression at glutamatergic synapses. More importantly, vGlut1-positive contacts onto granule cell dendrites were increased in cultures treated with all NMDAR antagonists except Ro25,6981 when compared to vehicle (Fig. 3A, B, D). These data suggest that increased excitatory synapse number is likely to account for increased mEPSC frequency in D-APV- and memantine-, but not Ro25,6981-treated slice cultures.

Presynaptic, synaptic, and extrasynaptic NMDAR function were altered differentially by chronic NMDAR blockade and partial inhibition of NR2B-containing NMDAR.

Presynaptic NMDAR. Activation of presynaptic NR2B-containing NMDARs can facilitate glutamate release and increase mEPSC frequency (Woodhall et al., 2001; Dalby and Mody, 2003; Sjostrom et al., 2003; Jourdain et al., 2007). Increased presynaptic NMDAR function and subsequent increased release probability could explain increased mEPSC frequency in Ro25,6981-treated cultures. To examine this possibility, we first measured mEPSC frequency before and after application of the $P2Y_1$ receptor agonist, MRS2365 in vehicle-treated cultures. $P2Y_1$ receptor activation in astrocytes increases astrocytic release of glutamate, which can then activate adjacent NMDARs. Activation of adjacent presynaptic NMDAR can increase neuronal glutamate release probability, presumably by presynaptic terminal depolarization (Jourdain et al., 2007). As expected, superfusion with MRS2365 significantly increased the frequency, but not the amplitude

of mEPSCs in granule cells from vehicle-treated cultures (Fig. 4). This effect was blocked by concurrent acute application of MRS2365 with the $P2Y_1$ receptor antagonist MRS2500 or the NR2B antagonist Ro25,6981 (Fig. 4A) illustrating its dependence on $P2Y_1$ receptor and NR2B-containing NMDAR activation, respectively.

Comparing the effects of chronic NMDAR antagonist treatment, we found that the MRS2365-induced increase in mEPSC frequency was more than doubled following chronic inhibition of NR2B-containing NMDAR with Ro25,6981, but was absent following chronic NMDAR blockade with D-APV (Fig. 4A). These data suggest that increased release probability due to elevated presynaptic NR2B-containing NMDAR function could account for increased mEPSC frequency in Ro25,6981-treated cultures. That said, MRS2365-induced increases in mEPSC frequency were occluded by addition of the uncompetitive NMDAR antagonist, MK-801 (1 mM) to the pipette solution (Fig. 4A). Inclusion of MK-801 in the pipette solution was used previously to distinguish between pre- and post-synaptic NMDAR activation (Nevian and Sakmann, 2006; Rodriguez-Moreno and Paulsen, 2008), which raises the possibility that increased MRS2365-induced increases in mEPSC frequency required activation of NR2B-containing NMDAR in the postsynaptic granule cell. However, MK-801 crosses the blood-brain barrier, presumably by diffusion across lipid membranes (Wieland et al., 1988; Stevens and Yaksh, 1990). Consequently, we cannot completely exclude the possibility that MK-801 diffused out of the postsynaptic neuron and blocked presynaptic NMDAR.

NMDAR-mediated mEPSCs. Because of the ambiguity associated with the MK-801 experiments, we next examined postsynaptic NMDAR function, starting with NMDAR-mediated mEPSCs ($mEPSCs_{NMDAR}$). Comparing the effects of chronic NMDAR antagonist treatment, we found that $mEPSC_{NMDAR}$ amplitude was reduced dramatically following chronic NMDAR blockade with D-APV, suggesting decreased functional expression of postsynaptic NMDAR. Additionally, $mEPSC_{NMDAR}$ frequency was decreased profoundly in granule cells from cultures treated chronically with D-APV (80%) or Ro25,6981 (46%) and (Table 1; Fig. 5B). Since reduced $mEPSC_{NMDAR}$ frequency occurred in treatment groups displaying increased mEPSC frequency and recording paradigms favoring NMDAR activation, these data suggested a dramatic reduction in the number of synapses containing functional NMDAR. These findings were surprising because previous immunohistochemical studies reported increased postsynaptic NMDAR clusters following chronic NMDAR blockade with APV (Rao and Craig, 1997; O'Brien et al., 1998). The most parsimonious explanations for the discrepancy include nonfunctional NMDAR, perisynaptically clustered NMDAR that were not activated by constitutive presynaptic glutamate release, nascent NMDAR-containing synapses without functional presynaptic elements (Liao et al., 1999) or reduced glutamate release probability at NMDAR only-containing synapses.

Further analyses of $mEPSC_{NMDAR}$ revealed that $mEPSC_{NMDAR}$ decay was decreased in all chronic NMDAR antagonist treatment groups, but was most pronounced following treatment with Ro25,6981 or D-APV (Table 1; Fig. 5D). These data suggest decreased synaptic localization of NR2B subunits, which convey a longer decay to NMDAR-mediated currents (Flint et al., 1997; Stocca and Vicini, 1998). Arguing against a global

decrease in synaptic NR2B-containing NMDAR, acute application of the NR2B-selective antagonist, Ro25,698 elicited similar small decreases in $mEPSC_{NMDAR}$ amplitude (Fig. 6C) and had no significant effect on $mEPSC_{NMDAR}$ decay for all chronic treatment groups (Fig. 6D), possibly due to difficulties in precisely defining decay times under low signal / noise conditions. However, while acute application of Ro25,6981 reduced $mEPSC_{NMDAR}$ frequency in cultures treated with vehicle (27%), memantine (43%) and NVP-AAM077 (43%), it had no effect in cultures treated with D-APV or Ro25,6981 (Fig.6B). These findings suggest that chronic treatment with D-APV or Ro25,6981 reduced the number of individual synapses containing functional NR2B-containing NMDAR.

Tonic NMDAR-mediated currents. Given this apparent reduction in individual synapses containing functional NR2B-containing NMDAR, we turned our attention to tonic NMDAR-mediated currents. Tonic NMDAR currents are mediated primarily by extrasynaptic NMDAR (Le Meur et al., 2007), which historically were thought to be comprised of NR2B-containing NMDARs (Sheng et al., 1994; Tovar and Westbrook, 1999). Under our recording conditions, tonic NMDAR-mediated currents were modest (<40 pA) (Suppl. Fig. 2). Therefore, tonic currents were enhanced with DL-TBOA (50 μM), a potent, highly selective competitive blocker of excitatory amino acid transporters. TBOA is not a neuronal transporter substrate and thus does not elicit transport currents or chloride fluxes (Shimamoto et al., 1998). Use of TBOA also eliminated confounds associated with potential alterations in transporter expression / function and the potentiating effects of Ro25,6981 at low glutamate concentrations (Kew et al., 1996; Fischer et al., 1997). TBOA-enhanced tonic currents were associated with a shift in

holding current, increased recording noise (Fig. 7 A, B) and superimposed slow inward currents (SICs) (Suppl. Fig. 3 – insets), all of which were blocked by acute application of D-APV (data not shown), Mg^{2+} (data not shown), or NVP-AAM007 (Fig. 7 A, B, data not shown), but unaffected by acute application of NR2B-selective antagonist Ro25,6981 (Fig. 7A, B; Suppl. Fig. 4) or specific blockade of neuronal glutamate release with tetanus toxin (Schiavo et al., 1992; Fellin et al., 2004). These findings showed that the shift in holding current, increased noise and superimposed slow inward currents (SICs) were mediated by non-NR2B-containing NMDAR and required astrocytic rather than neuronal glutamate release.

Comparison between treatment groups revealed that the percentage of cultures displaying SICs was similarly reduced in cultures treated chronically with all NMDAR antagonists (Table 3), suggesting that chronic NMDAR inhibition may decrease astrocytic glutamate release probability. Because SICs are thought to enable neuronal synchronization (Angulo et al., 2004; Fellin et al., 2004), these findings suggest that neuronal synchronization may be reduced following chronic treatment with NMDAR antagonists.

We also documented a trend toward decreased tonic NMDAR current amplitude in D-APV-treated cultures (Fig. 7C), consistent with previous immunohistochemical studies showing decreased extrasynaptic NMDAR following chronic NMDAR blockade (Rao and Craig, 1997) and a significant 78% increase in tonic NMDAR current amplitude following chronic inhibition of NR2B-containing NMDAR with Ro25,6981 (Fig. 7C). These changes were associated with a trend toward decreased open NMDAR channel number following chronic NMDAR blockade with D-APV- and a dramatic (154%)

increase following chronic inhibition of NR2B-containing NMDAR with Ro25,6981 when compared to vehicle (Fig. 7F). Increased channel number in Ro25,6981-treated cultures was accompanied by a significant, but less dramatic decrease in single NMDAR channel conductance (Fig. 7E). Although extrasynaptic NMDAR activation predominated in our recordings, synaptic NMDAR also were activated when glutamate transporters were blocked with TBOA (Suppl. Fig. 5). Thus, the dramatic reductions in synaptic NMDAR in D-APV-treated cultures (Table 1, Fig. 5) could account at least partially for trends toward decreased tonic current amplitude and NMDAR number (Fig. 7C, F). However, $mEPSC_{NMDAR}$ frequency was decreased and $mEPSC_{NMDAR}$ amplitude was only minimally increased in cultures treated chronically with Ro25,6981 (Table 1, Fig. 5), suggesting a dramatic increase in functional extrasynaptic NMDAR following chronic inhibition of NR2B-containing NMDAR with Ro25,6981.

Discussion

To our knowledge, this is the first report demonstrating changes in excitatory circuits following chronic inhibition of NMDAR with memantine, NR2B-containing NMDAR with Ro25,6981 and NR2A-containing NMDAR with NVP-AAM077. Our major findings for each NMDAR antagonist were as follows. Following *chronic NMDAR blockade with D-APV* we found increased mEPSC frequency, amplitude and charge transfer. Increased EPSC amplitude and charge transfer suggested increased postsynaptic AMPAR number and/or function. Increased EPSC frequencies together with a trend toward increased mossy fiber collaterals in the molecular layer and increased vGlut1-positive contacts onto granule cell dendrites, suggested increased excitatory synapse number. These pro-excitatory alterations likely contribute to the increased network excitability and seizures reported previously following chronic NMDAR blockade (O'Brien et al., 1998; McKinney et al., 1999; Wang and Bausch, 2004; Bausch et al., 2006). We also documented profoundly decreased $mEPSC_{NMDAR}$ frequency suggestive of decreased NMDAR-containing synapses.

Following *chronic NMDAR inhibition with memantine,* we found: 1) modestly increased mEPSC amplitude and charge transfer; 2) trend toward increased mossy fiber collaterals in the molecular layer; and 3) increased vGlut1-positive contacts onto granule cell dendrites. These rather limited pro-excitatory changes may explain in part why memantine can increase seizure susceptibility (Loscher, 1998; Wang and Bausch, 2004), but otherwise is generally well-tolerated clinically (Parsons et al., 1999).

Following *inhibition of NR2A-containing NMDAR with NVP-AAM077,* we found modestly increased mEPSC amplitude and charge transfer, a trend toward increased

mossy fiber collaterals in the molecular layer, and increased vGlut1-positive contacts onto granule cell dendrites. These findings are consistent with increased postsynaptic AMPAR number / function, increased excitatory synapse number and enhanced excitability.

Following *chronic inhibition of NR2B-containing NMDAR with Ro25,6981,* we documented decreased mossy fiber collaterals in CA3, a trend toward decreased mossy fiber length and in contrast to other NMDAR antagonists, no change in vGlut1-positive contacts onto granule cell dendrites. These data suggested modestly reduced synaptic reorganization and a role for NR2B-containing NMDAR in synaptic rearrangements. Additionally, these findings together with those from other NMDAR antagonists show an association between excitatory network reorganization and seizure expression (present study; Wang and Bausch 2004), consistent with a large body of literature on this topic (Khrestchatisky et al., 1995; Mody, 1999; Nadler, 2003).

We also documented decreased $mEPSC_{NMDAR}$ frequency suggestive of decreased NMDAR-containing synapses. These findings, together with similar albeit larger decreases in $mEPSC_{NMDAR}$ following chronic D-APV, suggest that changes in $mEPSC_{NMDAR}$ modulate individual synapses, but are not major contributors to the opposite effects of chronic D-APV and Ro25,6981 on global network excitability. We also found pro-excitatory changes, including increased mEPSC frequency, amplitude and charge transfer. However, similar changes were seen following chronic memantine and our previous work showed opposite effects of chronic memantine and Ro25,6981 on seizures. The dichotomy between EPSCs and seizures suggests that shifts in mEPSCs, which arise from a plethora of synaptic inputs onto individual neurons, may not

accurately predict changes in global network excitability or seizure expression and must be considered in the context of other circuit alterations.

Lastly, we documented increased tonic NMDAR current amplitude and channel number, which was specific for chronic Ro25,6981, but not other NMDAR antagonists, similar to our previous findings for seizure expression (Wang and Bausch, 2004). This association may provide clues for understanding the mechanism by which chronic NR2B antagonists reduce seizure expression.

The role of extrasynaptic NMDAR in neuronal excitability and network function. Changes in tonic NMDAR-mediated currents are difficult to place into the context of network function without a better understanding of the physiological role of extrasynaptic NMDAR, which mediate tonic NMDAR-mediated currents. Extrasynaptic NMDAR are activated following high frequency stimulation and subsequent synaptic glutamate spillover and / or astrocytic glutamate release. Previous reports suggested that tonic NMDAR-mediated currents activated by ambient endogenous glutamate levels elicited plateau potentials and enhanced neuronal excitability via increased excitatory input-output gain (Sah et al., 1989; Suzuki et al., 2008), which could lead to depolarization-induced block of action potential generation. However, other studies reported no significant effect of small amplitude tonic NMDAR-mediated currents on neuronal excitability (Cavelier and Attwell, 2005; Le Meur et al., 2007). Conversely, extrasynaptic NMDAR activation may lead to a shunting effect, analogous to the shunting inhibition seen following extrasynaptic $GABA_A$ receptor activation, which reduces temporal integration of excitatory synaptic inputs and suppresses action potential generation

(Brizzi et al., 2004; Semyanov et al., 2004; Cope et al., 2005; Farrant and Nusser, 2005). These seemingly disparate mechanisms are not mutually exclusive. Increased input-output gain may predominate at normal resting membrane potentials, while shunting could prevail when dendrites are depolarized to near the NMDAR reversal potential during periods of high network activity and seizures. Further studies that selectively modulate extrasynaptic NMDAR without affecting synaptic or presynaptic NMDAR are necessary to document the functional consequences of extrasynaptic NMDAR activation under diverse physiological and pathological conditions.

Role for changes in excitatory synapses in major depression.

Glutamate and NMDAR levels are upregulated in non-medicated patients with major depression (Vergara et al., 1974; Kim et al., 1982; Sanacora et al., 2004; Grant et al., 2009). A single dose of ketamine can significantly improve major depressive symptoms in refractory patients within hours of administration and persist for up to a week (Berman et al., 2000; Zarate et al., 2006). NR2B-selective antagonists show clinical efficacy against major depression without the dissociative effects associated with ketamine (Preskorn et al., 2008). The mechanisms by which NMDAR antagonists reduce major depressive symptoms remain unclear. However, animal studies showed that pretreatment with AMPAR antagonists attenuated ketamine-induced antidepressant behavior, suggesting that NMDA antagonists may exert their rapid antidepressant effects by increasing synaptic AMPAR / NMDAR ratios in critical neuronal circuits (Dybala et al., 2008; Maeng et al., 2008). Thus, the functional up-regulation of synaptic AMPAR

relative to synaptic NMDAR elicited by chronic blockade with all NMDAR antagonists may contribute to the clinical efficacy of NMDAR antagonists against major depression.

Potential role for altered astrocyte function in homeostatic NMDAR expression and disease.

We focused on the effects of chronic NMDAR inhibition on neuronal synapse and glutamate receptor function. However, our findings showing changes in extrasynaptic and perisynaptic NMDAR activated by astrocytic glutamate release raises the possibility that NMDAR antagonists differentially altered astrocytic function. NMDAR are expressed in astrocytes (Conti et al., 1996; Van Bockstaele and Colago, 1996; Lalo et al., 2006) and NR2B-containing NMDAR are up-regulated in astrocytic processes following CNS insults (Krebs et al., 2003). The functional role of astrocytic NR2B-containing NMDAR remains elusive. However, activation of astrocytic NMDAR contributes to glial proliferation and activation (Uchihori and Puro, 1993; Kato et al., 2006), which is a hallmark of human mesial temporal lobe epilepsy. These reactive astrocytes exhibit elevated Ca^{2+} signaling and enhanced glutamate release (Eid et al., 2008). Likewise, beginning at 4-10 DIV astrocytes in hippocampal slice cultures become hypertrophic and form a reactive "gliotic scar" covering the dentate gyrus, similar to that seen following injury in vivo (Coltman and Ide, 1996). Reduced astrocytic glutamate release secondary to chronic NMDAR inhibition could homeostatically increase extrasynaptic and perisynaptic NMDAR function. Arguing against this possibility, chronic treatment with all NMDAR antagonists similarly decreased SIC incidence, but these antagonists differentially altered extrasynaptic and perisynaptic NMDAR function. Thus, global

changes in SICs and by extension astrocytic glutamate release cannot fully account for functional changes.

Concomitant changes in other glial functions such as inflammation also could play a role in functional changes in neurons. Inflammation is one factor common to all disorders amenable to treatment with NR2B selective antagonists (Kadhim et al., 2008; McCoy and Tansey, 2008; Vezzani et al., 2008; Miller et al., 2009). Glia release cytokines, such as TNFα and interleukin-1β that can alter glutamate receptor and seizure expression (Furukawa and Mattson, 1998; Beattie et al., 2002; Wetherington et al., 2008) and mediate depressive symptoms (McNally et al., 2008). NMDAR antagonists reduce microglial TNF production and release (Shibakawa et al., 2005; Rosi et al., 2006) and block interleukin-1βs proconvulsive effects (Balosso et al., 2008). Thus, chronic treatment with NMDAR antagonists may alter the contribution of cytokines or other proinflammatory factors to seizure expression and other disease processes, independent of changes in synapse and glutamate receptor function.

Summary

We showed that chronic NMDAR inhibition altered neuronal circuitry, individual synapses and AMPAR and NMDAR function. These changes may contribute to the therapeutic efficacy of NMDAR antagonists. Conversely, they also may represent homeostatic changes that have limited effects on disease processes. Further studies are needed to differentiate between these possibilities.

Table 1. EPSCs in granule cells

Treatment	n	Frequency (Hz)	Amplitude (pA)	Charge Transfer (pA?ms)	Rise Time (ms)	Decay Time (ms)
mEPSCs						
Vehicle	18	0.35 ± 0.08	-16.9 ± 0.7	84 ± 6	2.8 ± 0.2	7.2 ± 0.3
D-APV	14	0.45 ± 0.05	-21.8 ± 0.9 [a, c]	124 ± 7 [a, c, d, e]	2.7 ± 0.1	8.5 ± 0.3 [a, c, d, e]
Memantine	20	0.36 ± 0.05	-16.7 ± 0.9	83 ± 6	2.5 ± 0.1	7.3 ± 0.3
Ro25,6981	25	0.49 ± 0.06	-18.1 ± 1.0	87 ± 6	2.5 ± 0.1	6.8 ± 0.2 [b, f]
NVP-AAM077	23	0.30 ± 0.03	-17.9 ± 1.0	89 ± 5	2.8 ± 0.1	7.2 ± 0.2
NVP+Ro	22	0.37 ± 0.03	-19.0 ± 0.7	103 ± 6	2.8 ± 0.1	7.8 ± 0.2
mEPSCs$_{NMDAR}$						
Vehicle	24	0.035 ± 0.003	-39.5 ± 1.9	2663	20.4 ± 0.4	162 ± 10 (15)
D-APV	23	0.007 ± 0.001 [a, c, d, e]	-28.0 ± 0.9 [a, c, d, e, f]	1340	19.4 ± 0.9	113 ± 8 [a] (15)
Memantine	19	0.037 ± 0.004	-37.1 ± 1	3533	20.2 ± 0.5	130 ± 7 [a, d] (15)
Ro25,6981	21	0.019 ± 0.003 [a, b, c, e]	-38.3 ± 1.4	1741	17.8 ± 0.5 [a, e]	98 ± 7 [a] (15)
NVP-AAM077	26	0.037 ± 0.004	-37.8 ± 1.0	3209	20.8 ± 0.4	134 ± 5 [a, d] (15)
NVP+Ro	19	0.017 ± 0.002 [a, c, e]	-38.9 ± 1.5	2542	18.3 ± 0.4 [e]	92 ± 5 [a, e] (15)

Table 1. EPSCs in granule cells. Means ± SEM, except charge transfer of mEPSCsNMDAR, which was measured from the averaged trace from all events. Abbreviations: n, the number of granule cells / hippocampal slice cultures investigated. [a], different than vehicle; [b], different than D-APV; [c], different than memantine; [d], different than Ro25,6981; [e], different than NVP-AAM077; [f], different than NVP+Ro; $p<0.05$, ANOVA with Holm-Sidak post hoc comparison. See Figures 1 and 5 for representative traces and cumulative probability plots.

Table 2. Granule cell morphology

Length (µm)					Number			
Total	ML	GCL	Hilus	CA3	Branch Points	Ends	Boutons	Boutons/µm axon
6091 ± 384	12 ± 6	515 ± 78	3472 ± 338	2092 ± 269	33.4 ± 2.8	37.2 ± 3.2	235 ± 21	0.039 ± 0.003
6540 ± 450	45 ± 22	513 ± 106	3689 ± 391	2293 ± 260	35.0 ± 2.5	39.3 ± 2.7	270 ± 18	0.042 ± 0.002
5446 ± 253	24 ± 15	646 ± 115	3020 ± 217	1756 ± 125	29.5 ± 1.8	33.6 ± 2.2[e]	222 ± 17	0.041 ± 0.003
5171 ± 336[b]	14 ± 11	582 ± 139	3374 ± 296	1201 ± 152[a, b]	26.7 ± 2.0[e]	30.0 ± 2.4[e]	220 ± 24	0.043 ± 0.004
5924 ± 204	30 ± 17	640 ± 98	3241 ± 175	2013 ± 197	38.0 ± 2.1	44.4 ± 2.5	251 ± 15	0.043 ± 0.002
5094 ± 224[b]	8 ± 7	594 ± 105	2871 ± 289	1621 ± 214	28.2 ± 2.0[e]	32.8 ± 2.3[e]	238 ± 14	0.047 ± 0.002
1623 ± 86	862 ± 110	731 ± 78	9.7 ± 6.7	NA	13.8 ± 0.8	16.7 ± 0.8	NA	NA
1874 ± 75	834 ± 102	1030 ± 116	3.5 ± 2.4	NA	13.4 ± 0.7	16.9 ± 0.5	NA	NA
1803 ± 79	910 ± 101	892 ± 124	0.4 ± 0.4	NA	14.5 ± 0.9	17.6 ± 0.9	NA	NA
1829 ± 49	829 ± 91	955 ± 104	3.8 ± 3.8	NA	13.9 ± 0.7	17.4 ± 0.7	NA	NA
1875 ± 87	902 ± 93	947 ± 110	4.3 ± 4.0	NA	13.5 ± 0.6	16.8 ± 0.7	NA	NA
1727 ± 80	944 ± 109	776 ± 101	6.3 ± 5.9	NA	13.4 ± 0.6	16.3 ± 0.6	NA	NA

Means ± SEM. *Abbreviations*: ML, molecular layer; GCL, granule cell layer; NA, not applicable. n, the number of granule cells / hippocampal slice cultures investigated. [a], different than vehicle; [b], different than D-APV; [e], different than NVP-AAM077; $P<0.05$, ANOVA with Holm-Sidak *post hoc* comparison. See Figure 1 for representative granule cell morphology.

Table 3. Slow inward currents superimposed on the NMDAR-mediated tonic current in granule cells

Treatment	n	Frequency (SIC/min)	Amplitude (pA)	Rise Time (ms)	Decay Time (ms)	Charge Transfer (pA·ms) × 10^3	SIC incidence
Vehicle	20	0.50 ± 0.10	-68.8 ± 10.8	399 ± 61	1265 ± 232	154.5 ± 32.1	20/22
D-APV	13	0.35 ± 0.08	-51.1 ± 5.5	389 ± 93	1059 ± 257	127.0 ± 37.5	13/23[a]
Memantine	9	0.26 ± 0.06	-72.4 ± 13.4	267 ± 40	911 ± 143	140.0 ± 37.2	9/15[a]
Ro25,6981	8	0.18 ± 0.05	-93.2 ± 16.1	338 ± 73	1031 ± 240	184.3 ± 46.2	8/21[a]
NVP-AAM077	11	0.32 ± 0.07	-122.8 ± 33.9	318 ± 77	1049 ± 234	175.4 ± 31.8	11/22[a]
NVP+Ro	5	0.40 ± 0.14	-66.3 ± 30.5	574 ± 69	1582 ± 104	171.6 ± 56.7	5/18[a]

Table 3. Slow inward currents. Means ± SEM. *Abbreviation*: SIC, slow inward currents. n, the number of granule cells / hippocampal slice cultures investigated. SIC incidence = cultures displaying slow inward NMDAR-mediated currents superimposed on the tonic current / total cultures investigated. a, different than vehicle; $p < 0.05$, Chi-square test.

Figure 1. mEPSCs

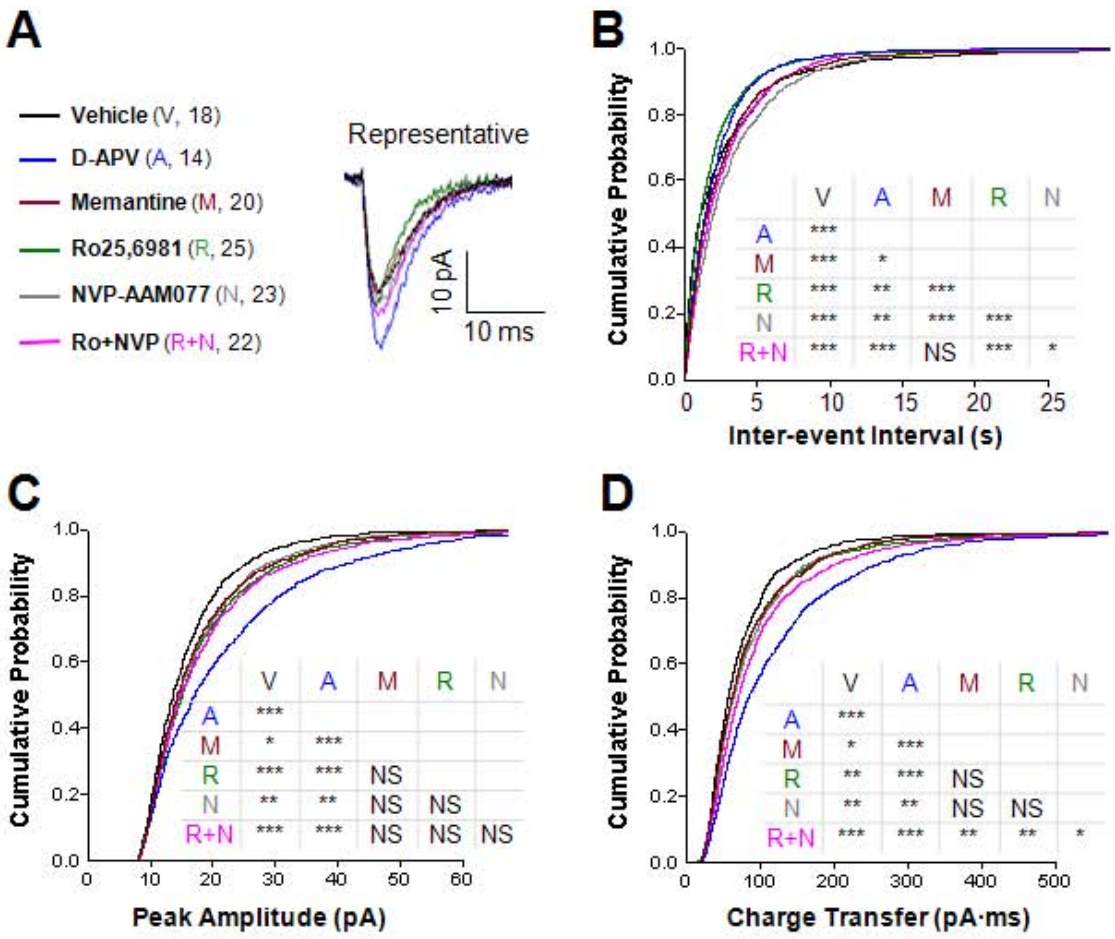


Figure 1. Miniature EPSCs in granule cells were dramatically enhanced in D-APV-treated hippocampal slice cultures, while intermediate effects were noted in cultures treated with the other NMDAR antagonists. Recordings were conducted at a -70 mV holding potential in physiological recording buffer containing TTX (1 μM) and BMI (10 μM). ***A,*** Averages of mEPSCs from representative granule cells illustrate increased amplitude for D-APV relative to vehicle and all other NMDAR antagonists. Cumulative probability plots show increases in mEPSC ***B,*** frequency in granule cells from slice cultures treated with Ro25,6981 and D-APV and modest differences in the other treatment groups. Large increases in ***C,*** peak amplitude and ***D,*** charge transfer were apparent following treatment with D-APV; intermediate increases were seen following treatment with the other NMDAR antagonists. ***Insets,*** Tables show statistical comparisons between treatment groups. * $p \leq 0.025$; ** $p \leq 0.005$; *** $p \leq 0.0000$; NS, not significant; two-tailed Kolmogorov-Smirnov test. Legend in A applies to A-D; the number of granule cells / slice cultures is indicated in parentheses. See Table 1 for means.

Figure 2. granule cell morphology

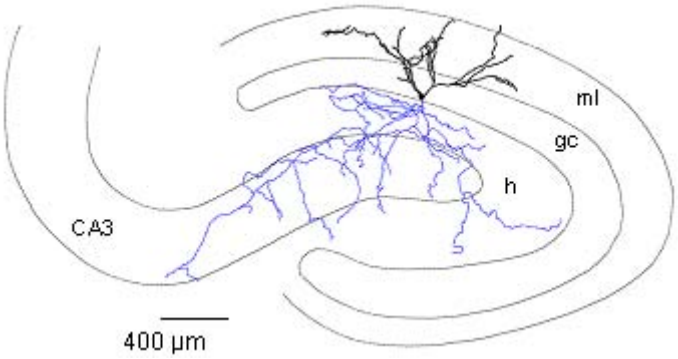


Figure 2. Morphology of a representative neurobiotin-filled granule cell from a vehicle-treated hippocampal slice culture. *Abbrev.:* ml, molecular layer; gcl, granule cell layer; h, hilus. Thick black lines, dendrites; blue lines, axons; thin black lines, region borders. See Table 3 for quantitative analyses.

Figure 3. vGlu1-positive contacts

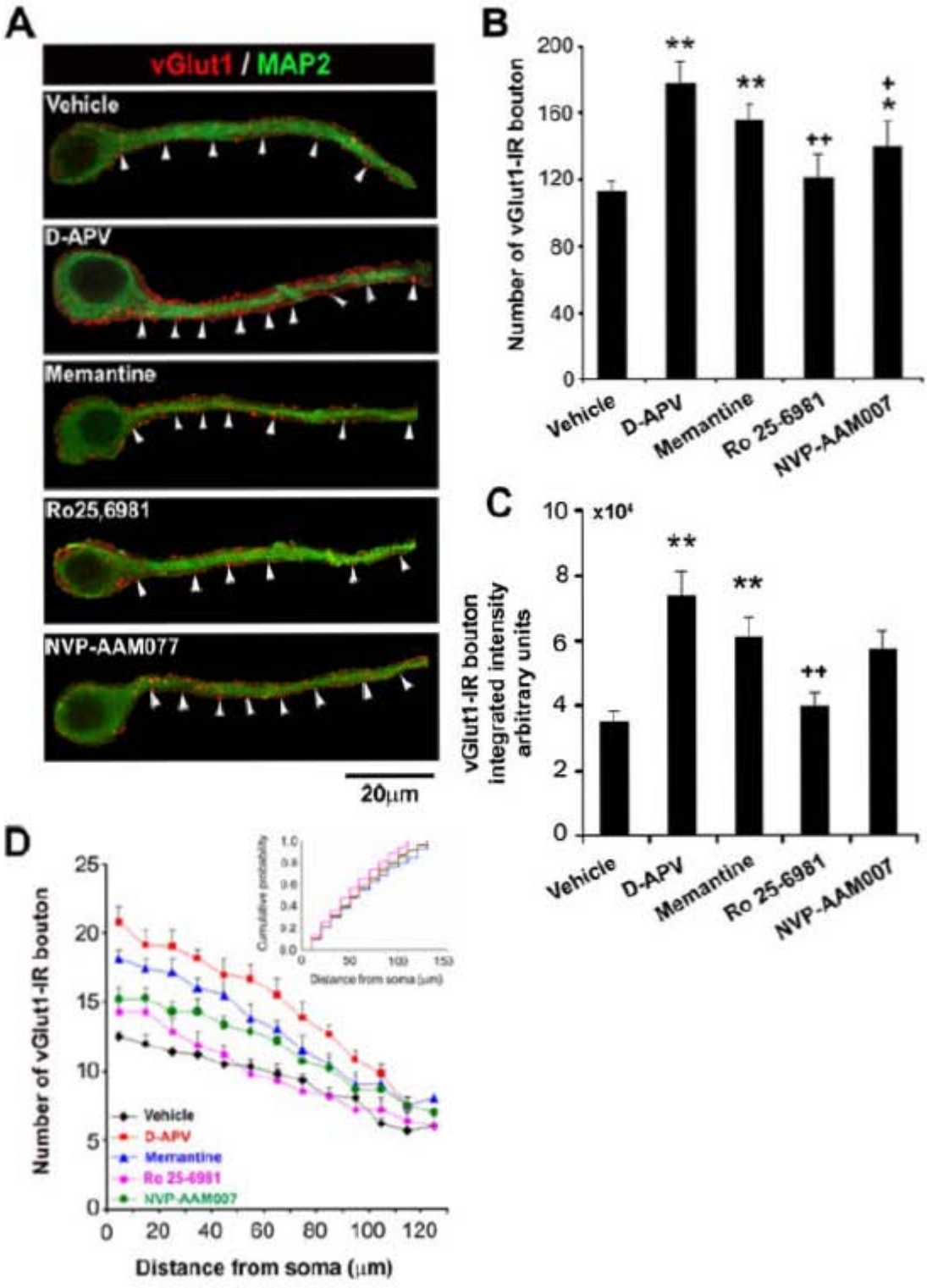


Figure 3. The number and intensity of vGlut1-positive excitatory synapses onto granule cell dendrites were increased in hippocampal slice cultures treated with all NMDAR antagonists except Ro25,6981. Cultures were double-labeled for vGlut1 (red, for presynaptic glutamatergic terminals) and MAP2 (green, for dendrites) using immunofluorescence. ***A,*** Single optical sections from representative granule cells illustrate vGlut1 immunoreactive puncta (arrowheads) along a primary apical dendrite. Yellow indicates overlap of vGlut1 and MAP2 immunoreactivities. Surrounding elements were subtracted digitally for clarity. The ***B,*** number and ***C,*** integrated intensity of vGlut1-IR puncta apposed to single granule cell dendrites were increased in chronic treatment with all NMDAR antagonists except Ro25,6981. ***D:*** Plots of vGlut1-IR puncta distribution (10 μm bins) along apical granule cell dendrites substantiated compiled means. ***D inset:*** Cumulative probability plots showed no significant differences in the distribution of vGlut1-IR puncta along granule cell dendrites ($p>0.025$, two-tailed Kolmogorov-Smirnov test). Data were collected from 3-4 granule cell dendrites / slice culture in 5-7 individual slice cultures / independent experiments. *$p< 0.05$, **$p< 0.01$, different than vehicle; ++, $p<.001$ different than APV; Kruskal Wallis ANOVA by Ranks with Dunn's post hoc comparison. *Scale bar* in *A* applies to all panels in A. (Work done by Dr. Yu Wang)

Figure 4. Presynaptic NMDAR

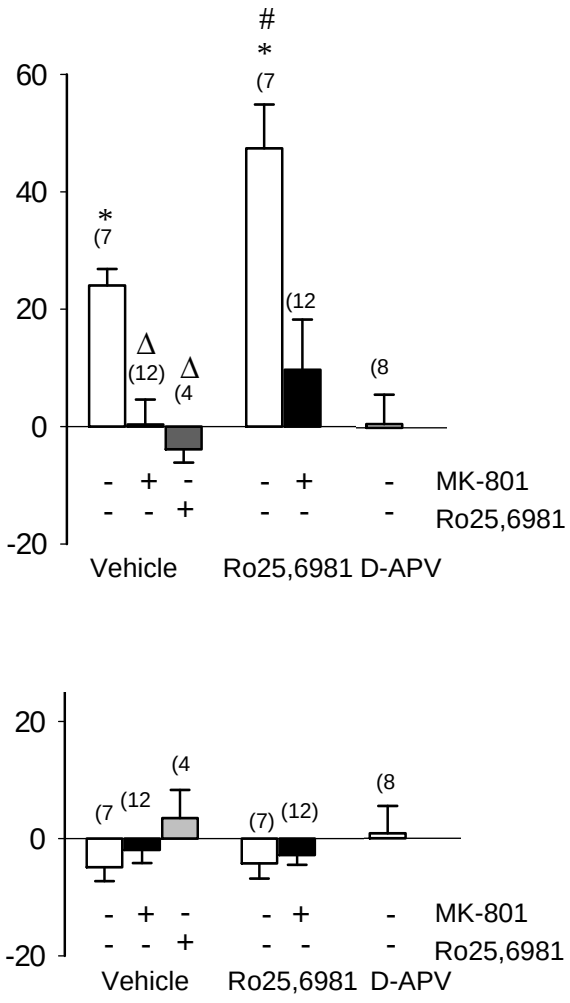


Figure 4. Putative presynaptic NR2B-containing NMDARs were functionally up-regulated in excitatory synapses onto granule cells in hippocampal slice cultures treated with Ro25,6981. Recordings of mEPSCs in dentate granule cells were conducted at a -70 mV holding potential in 0 mM Mg^{2+} recording buffer containing TTX (1 μM) and BMI (10 μM) for at least 5 min. before, 2 min. during and 7 min. after application of the $P2Y_1$ antagonist, MRS2365 (3 μM). ***A,*** Application of MRS2365 significantly increased mEPSC frequency in vehicle- and to a much larger extent in Ro25,6981-treated slice cultures. This increase was blocked by acute bath application of Ro25,6981(1 μM) or inclusion of MK-801 (1 mM) in the pipette solution. ***B,*** Miniature EPSC amplitude remained unaltered. * $p<0.01$, different than before MRS2365 application, paired t-test; $^{\Delta}$ $p<0.01$, different than no MK-801 / no acute Ro25,6981 within the chronic vehicle or Ro25,6981 treatment groups (white bars), t-test; $^{\#}$ $p<0.01$, different than vehicle, t-test. The number of granule cells / slice cultures is indicated in parentheses.

Figure 5. NMDAR-mediated mEPSCs

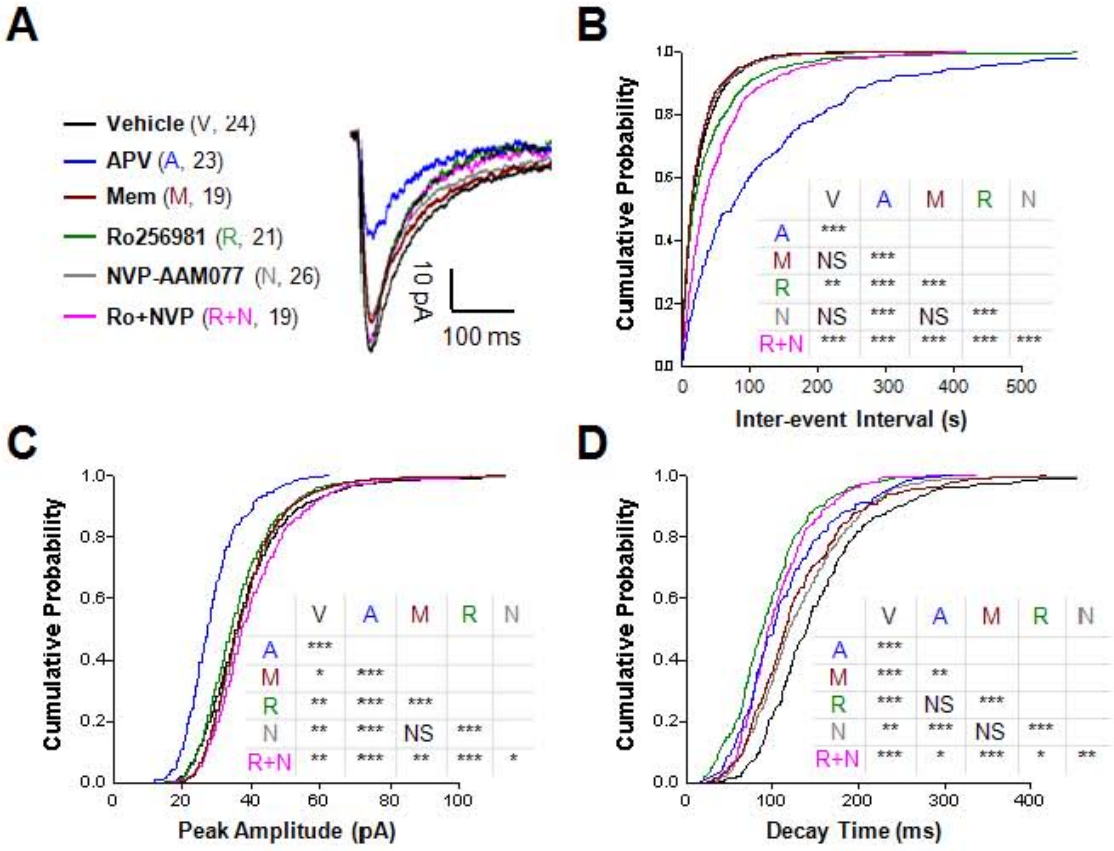


Figure 5. NMDA receptor-mediated mEPSCs ($mEPSC_{NMDAR}$) in granule cells were dramatically reduced in D-APV- and Ro25,6981-treated hippocampal slice cultures, but only modestly altered in cultures treated with the other NMDAR antagonists. Recordings were conducted at a -45 mV holding potential in 0 mM Mg^{2+} recording buffer containing TTX (1 μM), BMI (10 μM) and NBQX (10 μM). **A,** Averages of all $mEPSC_{NMDAR}$ from representative granule cells illustrate decreased amplitude for D-APV relative to vehicle and all other NMDAR antagonists. Cumulative probability plots show that $mEPSC_{NMDAR}$ ***B,*** frequency and ***C,*** amplitude were dramatically reduced in granule cells from D-APV-treated cultures; smaller decreases in frequency were seen for Ro25,6981. ***D,*** Miniature $EPSC_{NMDAR}$ decay times were decreased in all NMDAR antagonist treatment groups, but were most prominent for Ro25,6981 and D-APV. ***Insets,*** Tables show statistical comparisons between treatment groups. * $p \leq 0.025$; ** $p \leq 0.005$; *** $p \leq 0.0000$; NS, not significant; two-tailed Kolmogorov-Smirnov test. Legend in A applies to A-E. The number of granule cells / slice cultures is indicated in parentheses, except for D, where decays were measured for 20 events/cell in 15 granule cells (slice cultures) / treatment group. See Table 1 for means.

Figure 6. NR2 subunit-mediated mEPSCs$_{NMDAR}$

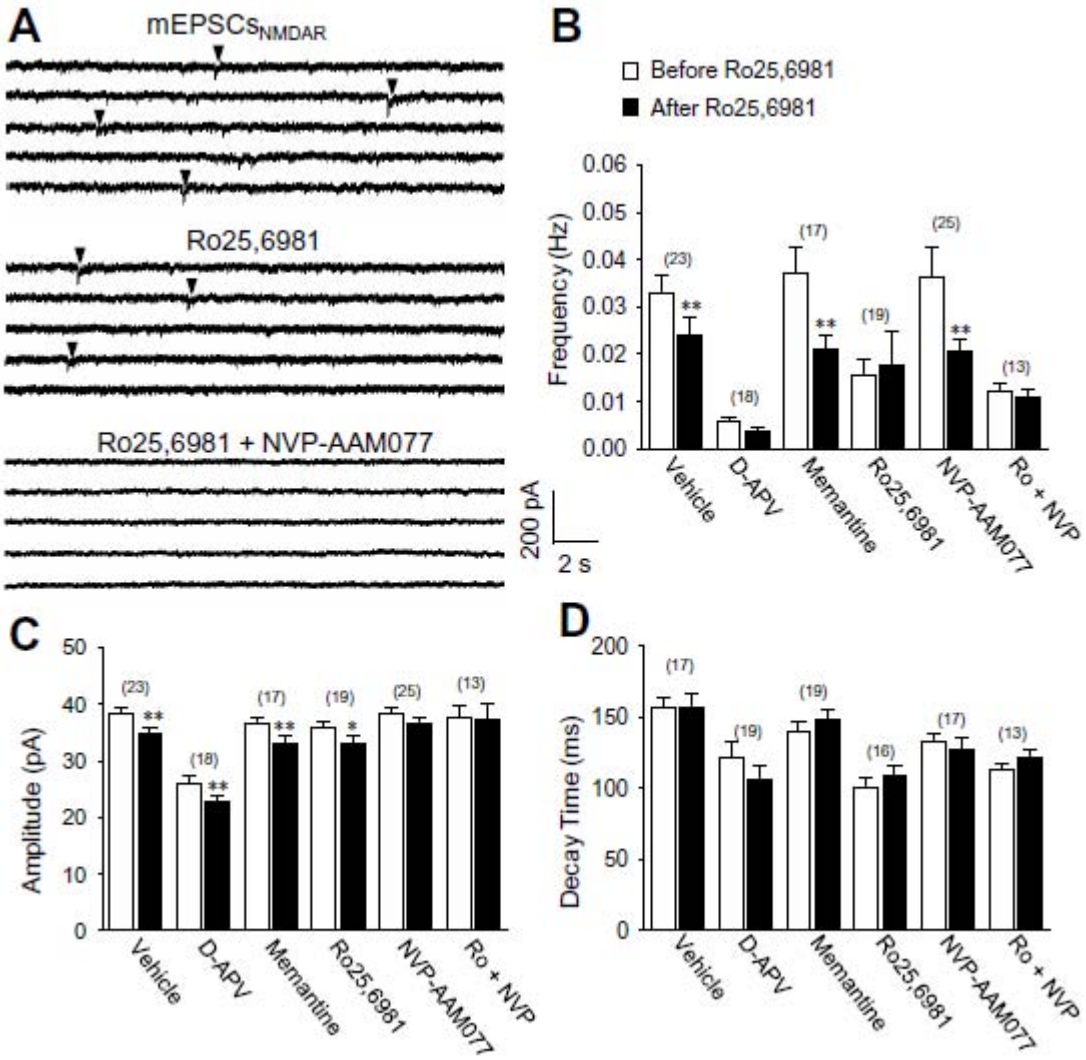


Figure 6. Miniature EPSCs$_{NMDAR}$ in granule cells from hippocampal slice cultures were mediated predominantly by NR2A-containing NMDAR. Recordings were conducted at a -45 mV holding potential in 0 mM Mg^{2+} recording buffer containing TTX (1 μM), BMI (10 μM) and NBQX (10 μM). ***A,*** Traces illustrate representative mEPSC$_{NMDAR}$ ***top***, before acute application of Ro25,6981, ***middle***, after acute application of Ro25,6981 (1 μM), and ***bottom,*** after addition of NVP-AAM077 (0.5 μM) to buffer containing Ro25,6981 in a vehicle-treated slice culture; arrowheads indicate mEPSCs$_{NMDAR}$. Bar graphs show significant decreases in ***B,*** frequency and ***C,*** amplitude, but not ***D,*** decay times for mEPSCs$_{NMDAR}$ following acute application of Ro25,6981. * $p<0.05$; ** $p\leq0.01$, different than before acute Ro25,6981 application, paired t-test. Legend in B is for B-D. The number of granule cells/slice cultures is indicated in parentheses.

Figure 7. Tonic NMDAR-mediated currents

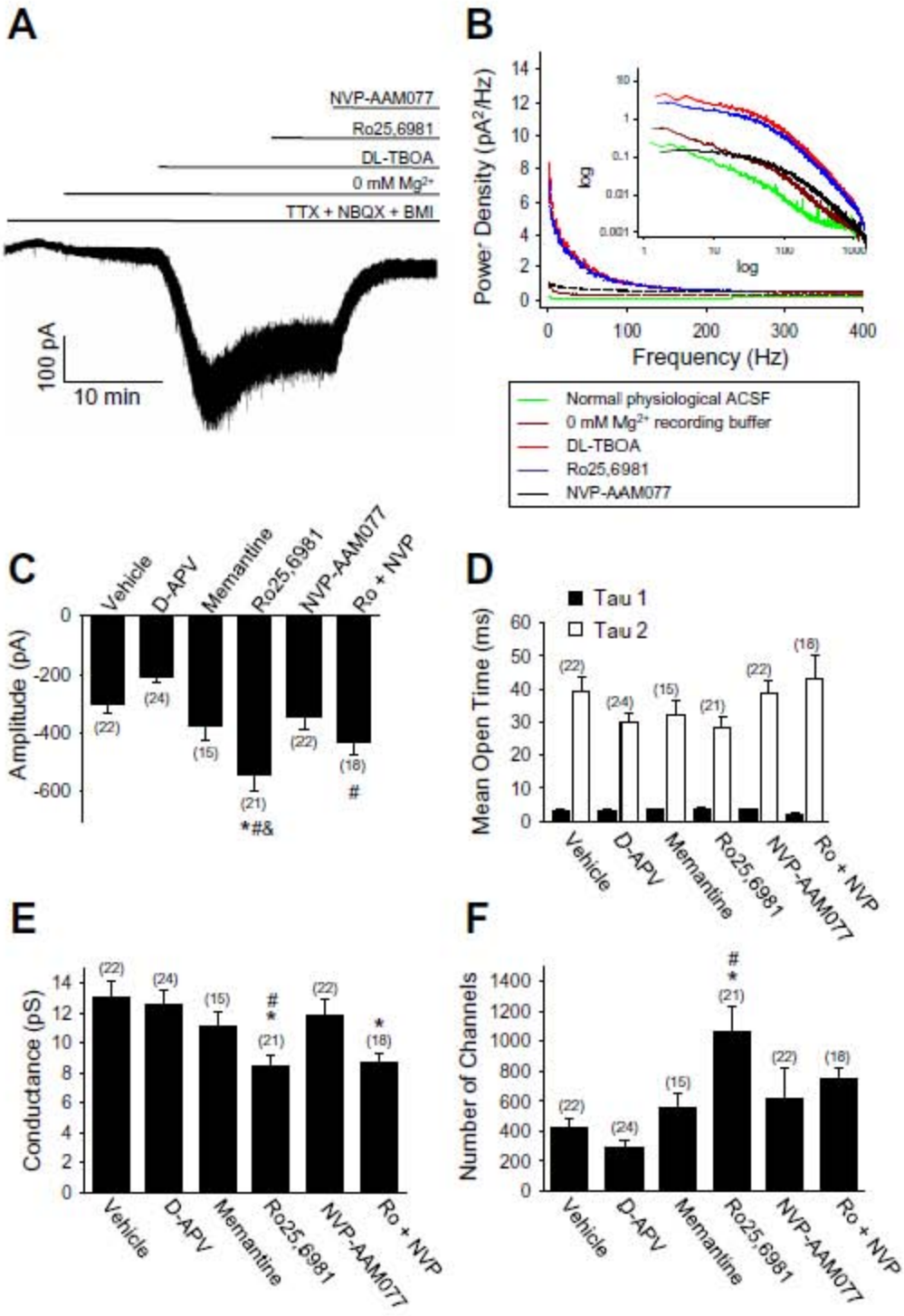
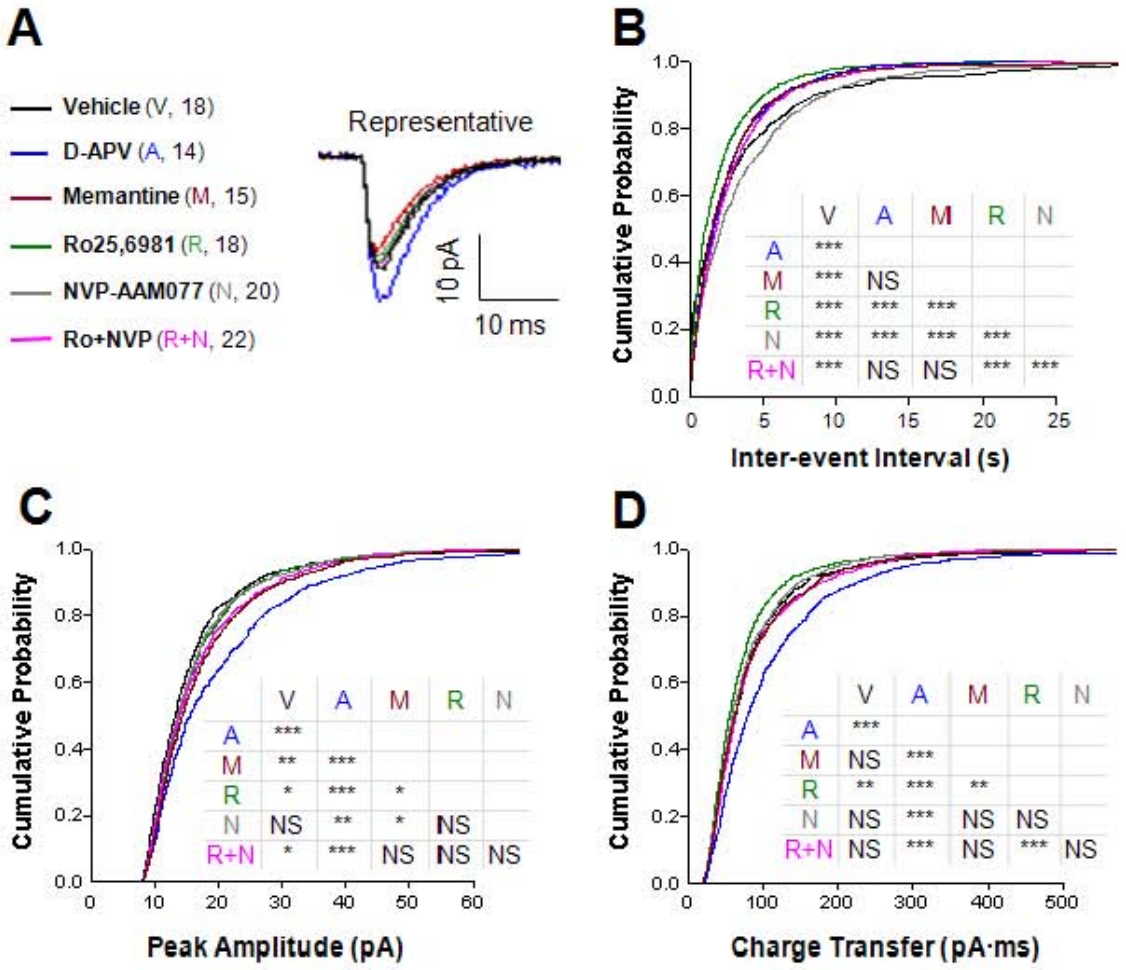


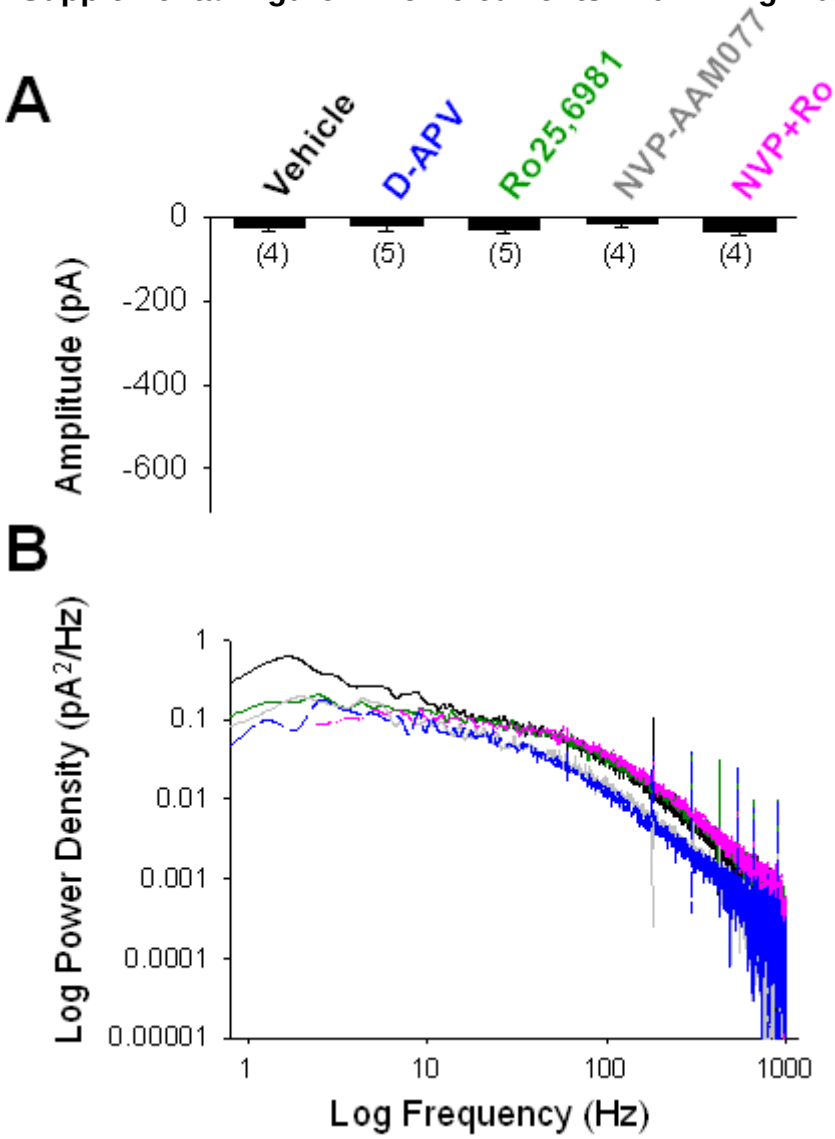
Figure 7. Tonic NMDA receptor-mediated currents and NMDAR channel number were increased in granule cells from Ro25,6981-treated hippocampal slice cultures. Recordings were conducted at a -70 mV holding potential in 0 mM Mg^{2+} recording buffer containing DL-TBOA (50 μM), TTX (1 μM), BMI (10 μM) and NBQX (10 μM). Representative ***A,*** trace and ***B,*** power spectra of a tonic NMDA receptor-mediated current recorded in a granule cell from a vehicle-treated slice culture. Note the change in holding current and increase in noise associated with application of 0 mM Mg^{2+} recording buffer that was accentuated by addition of the glutamate reuptake inhibitor, TBOA, and reversed by acute application of Ro25,6981(1 μM) + NVP-AAM077 (0.5 μM) but not Ro25,6981 (1 μM) alone. Compiled data show significantly increased ***C,*** tonic NMDAR-mediated current amplitude (maximal change in holding current) in cultures treated with Ro25,6981. Noise analyses revealed ***F,*** increased NMDAR channel number, ***E,*** decreased single NMDAR channel conductance, ***D,*** but unaltered mean open time (τ_{fast}, τ_{slow}) in granule cells from Ro25,6981-treated slice cultures compared to vehicle. *, different than vehicle; #, different than D-APV; &, different than NVP-AAM077; $p<0.05$, ANOVA with Holm-Sidak *post hoc* comparison. The number of granule cells/slice cultures is indicated in parentheses.

Supplemental Figure 1. AMPAR-mediated mEPSCs



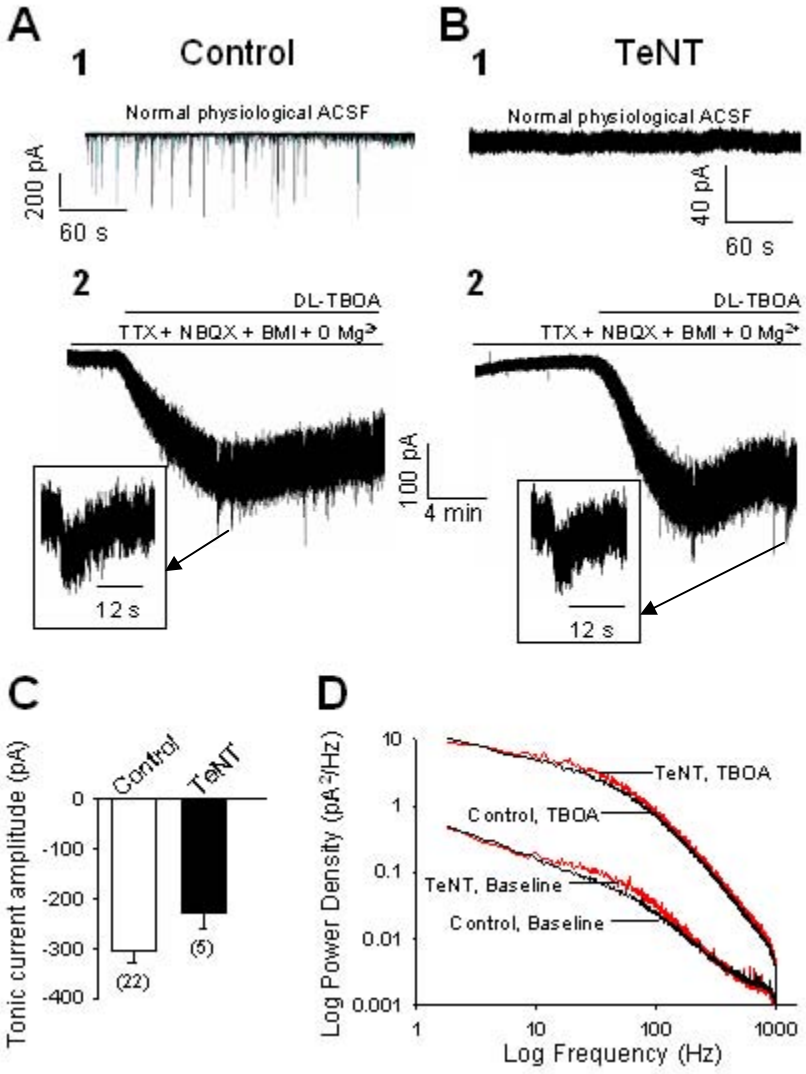
Supplemental Figure 1. AMPA receptor-mediated mEPSCs ($mEPSCs_{AMPAR}$) in granule cells were enhanced in D-APV-treated hippocampal slice cultures, but only modestly altered in cultures treated with the other NMDAR antagonists. Recordings were conducted for 160s at a -70 mV holding potential in physiological recording buffer containing TTX (1 μM), BMI (10 μM) and D-APV (50 μM). ***A,*** Averages of all $mEPSCs_{AMPAR}$ from representative granule cells illustrate increased amplitude for D-APV relative to vehicle and all other NMDAR antagonists. Cumulative probability plots revealed increased $mEPSCs_{AMPAR}$ ***B,*** frequency in all treatment groups except NVP-AAM077. Large increases in ***C,*** peak amplitude and ***D,*** charge transfer were apparent in granule cells from slice cultures treated with D-APV; modest or no differences were seen in the other treatment groups. ***Insets,*** Tables show statistical comparisons between treatment groups. * $p \leq 0.025$; ** $p \leq 0.005$; *** $p \leq 0.0000$; NS, not significant; two-tailed Kolmogorov-Smirnov test. Legend in A applies to A-F; the number of granule cells/slice cultures is indicated in parentheses.

Supplemental Figure 2. Tonic currents in 0mM Mg^{2+} buffer



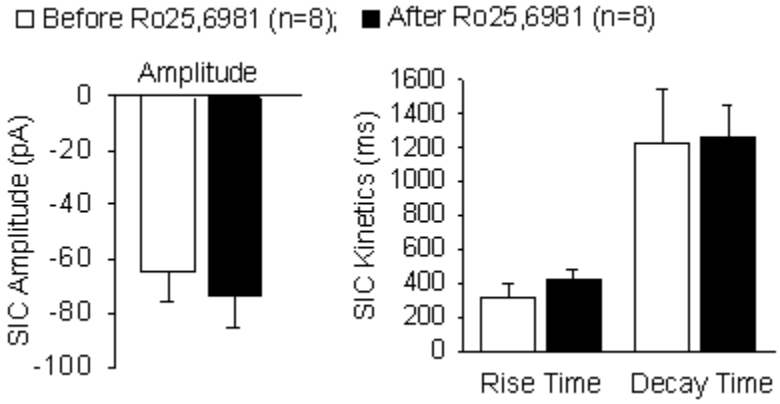
Supplemental Figure 2. Under our recording conditions, tonic NMDAR-mediated currents were modest in the absence of glutamate uptake blockers. Recordings of tonic NMDA receptor-mediated currents were conducted at a holding potential of -70 mV in 0 Mg^{2+} recording buffer containing TTX (1 μM), BMI (10 μM) and NBQX (10 μM). Compiled data revealed no significant changes in ***A,*** amplitude or ***B,*** spectral power density of the noise associated with tonic NMDAR-mediated currents induced by 0 Mg^{2+}. Line color in ***B*** is consistent with label color for treatments in ***A***. The number of granule cells/slice cultures is indicated in parentheses.

Supplemental Figure 3. Glutamate origin



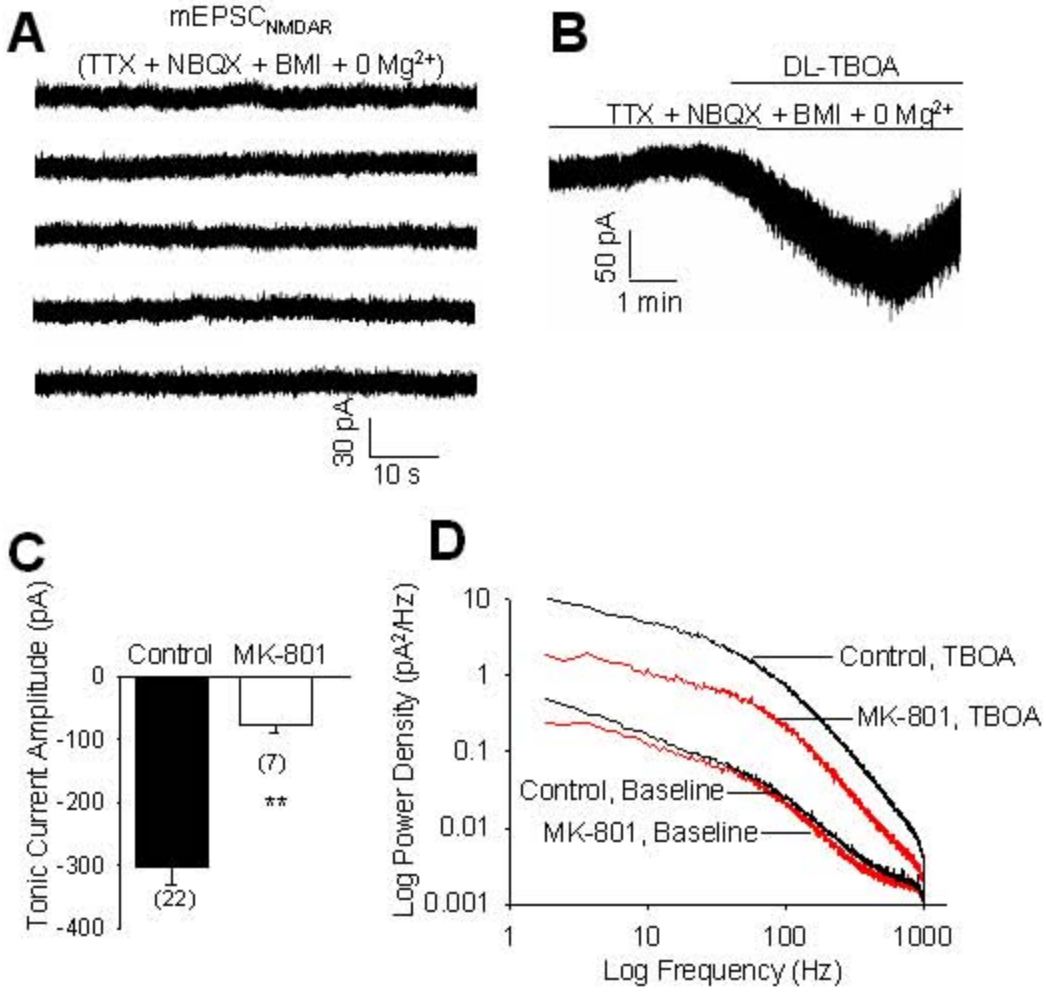
Supplemental Figure 3. NMDA receptor-mediated tonic currents and slow inward currents (SIC) in granule cells were mediated by glutamate released from glia. Recordings were conducted at a -70 mV holding potential. Tetanus toxin (TeNT, 2 μM; Sigma) was diluted in culture medium and cultures were immersed in TeNT-containing medium for 2 hr. prior to recordings. Representative traces show ***A1,*** spontaneous postsynaptic currents (sPCSs) recorded in physiological recording buffer and ***A2,*** a tonic NMDAR-mediated current recorded in 0 mM Mg^{2+} recording buffer containing TTX (1 μM), BMI (10 μM) and NBQX (10 μM) following addition of DL-TBOA (50 μM) in the same granule cell from a vehicle-treated slice culture. ***A2 inset,*** shows an expanded time scale of a SIC superimposed on the tonic NMDAR-mediated current. Representative traces recorded after 2 hr. incubation with tetanus toxin (TeNT, 2 μM) show an ***B1,*** absence of sPCSs, but ***B2,*** an intact tonic NMDAR-mediated current and ***B2 inset,*** SIC recorded in the same granule cell from a vehicle-treated slice culture. Compiled data reveal no significant effect of TeNT on the ***C,*** amplitude or ***D,*** spectral power density of the tonic NMDAR-mediated currents. The number of granule cells/slice cultures is indicated in parentheses.

Supplemental Figure 4. Slow inward currents



Supplemental Fig. 4. NMDA receptor-mediated slow inward currents (SIC) in granule cells did not involve NR2B subunits. Recordings were conducted in 0 Mg^{2+} buffer containing TTX (1 μM), BMI (10 μM), NBQX (10 μM) and DL-TBOA (50 μM) at a holding potential of -70 mV. Compiled data reveal no significant effect of acute blockade of NR2B-containing NMDARs (Ro25,6981, 1 μM) on SIC ***left,*** amplitude or ***right,*** rise and decay time in granule cells from vehicle-treated slice cultures. The number of granule cells/slice cultures is indicated in parentheses.

Supplemental Figure 5. Tonic currents mediated by extrasynaptic NMDAR



Supplemental Figure 5. Tonic NMDAR currents were mediated at least in part by extrasynaptic NMDA receptors. Slice cultures were incubated in physiological recording buffer containing BMI (10 μM) and (+)-MK-801 hydrogen maleate (MK-801, 10 μM; Sigma) for 10 min. Recordings were then conducted 20 min after washout of MK-801 in 0 Mg^{2+} recording buffer containing 1 μM TTX, 10 μM BMI and 10 μM NBQX. Representative traces ***A,*** recorded at a holding potential of -45 mV showed a lack of $mEPSC_{NMDAR}$, but an intact ***B,*** tonic NMDAR-mediated current recorded at a holding potential of -70 mV in the same granule cell from a vehicle-treated slice culture. Compiled data show that the ***C,*** amplitude and ***D,*** spectral power density of the noise associated with tonic NMDAR-mediated currents was reduced, but not completely blocked by MK-801. Arguing against MK-801 blocking only synaptic NMDAR Harris and Pettit (2008) showed that stimuli at as low as 25 Hz produced substantial extrasynaptic NMDAR activation. This raises the possibility that BMI-induced neuronal firing led to activation extrasynaptic NMDARs, which were blocked by MK-801. **, $p<0.01$, t-test; the number of granule cells/slice cultures is indicated in parentheses.

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Chapter 3

Enhanced Kv1 channel function contributes to reduced sIPSC frequency following chronic inhibition of NR2B-containing NMDAR with Ro25,6981.

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Abstract

Numerous studies have documented the effects of chronic NMDAR blockade on excitatory circuits, but the effects on inhibitory circuitry are not well-studied. NR2A- and NR2B-containing NMDAR play differential roles in physiological processes, but the consequences of chronic inhibition of NR2A- or NR2B-containing NMDAR on glutamatergic and GABAergic neurotransmission are unknown. In this study we documented alterations in GABAergic neurotransmission in dentate granule cells and interneurons following chronic treatment with the NR2B-selective antagonist, Ro25,6981, the NR2A-prefering antagonist, NVP-AAM077 or the non-subunit-selective NMDAR antagonist, D-APV in organotypic hippocampal slice cultures. Whole-cell voltage-clamp recordings revealed that the average sIPSC frequency was reduced dramatically in both granule cells and interneurons following chronic inhibition of NR2B-containing NMDAR with Ro25,6981 and was associated with no change in mean sIPSC amplitude, mIPSC frequency or mIPSC amplitude, suggesting diminished action potential-dependent GABA release. Chronic treatment with NVP-AAM077 or D-APV had no significant effect on any of these measures. Reduced sIPSC frequency did not arise from down-regulated $GABA_AR$, decreased excitatory or increased inhibitory drive to interneurons, altered interneuron membrane properties, increased failure rate, decreased action potential-dependent release probability, or mGluR/$GABA_B$ receptor modulation of GABA release. However, chronic Ro25,6981-mediated reductions in sIPSC frequency were occluded by dendrotoxin, margatoxin, agitoxin, but not dendrotoxin-K or XE991, suggesting that increased Kv1 channel function contributed to diminished action potential-dependent GABA release following chronic inhibition of NR2B-containing NMDAR with

Ro25,6981 and that these Kv1 channels may be heteromeric complexes containing Kv1.3, Kv1.6 and possibly Kv1.2.

Introduction

N-methyl-D-aspartate receptors (NMDAR) are heteromeric ionotropic glutamate receptors composed of NR1 and NR2A-D, with NR2A and NR2B being the predominant subunits in hippocampus and cortex (Monyer et al., 1994; Yamakura and Shimoji, 1999). NMDAR activation plays a crucial role in physiological brain processes, including neuronal survival (Ikonomidou et al., 1999) and synaptic plasticity (Mori and Mishina, 1995; Dingledine et al., 1999). Chronic NMDAR blockade increases synaptic reorganization in glutamatergic circuits, presynaptic glutamate release, and postsynaptic glutamate receptor clustering (Cline et al., 1987; Bear et al., 1990; Rao and Craig, 1997; Lin and Constantine-Paton, 1998; O'Brien et al., 1998; McKinney et al., 1999; Bausch et al., 2006). Such changes increase neuronal excitability and may exacerbate electrographic seizures following chronic NMDAR blockade (Bausch et al., 2006).

NMDAR also are highly expressed in hippocampal interneurons (Moriyoshi et al., 1991; Monyer et al., 1994), where they regulate inhibitory synapse plasticity (Xie and Lewis, 1995; Lu et al., 2000; Ouardouz and Sastry, 2000), postsynaptic $GABA_A$ receptor ($GABA_AR$) membrane insertion (Xie and Lewis, 1995; Marsden et al., 2007) and presynaptic GABA release (Drejer et al., 1987; Pin et al., 1988). Chronic NMDAR blockade alters $GABA_AR$ subunit expression (Matthews et al., 2000) and slightly reduces miniature inhibitory postsynaptic currents (mIPSC) (Bausch et al., 2006), but overall, documentation of chronic NMDAR blockade effects on inhibitory interneurons lags far behind that for excitatory circuits.

NR2A- and NR2B-containing NMDAR differ in subcellular localization, trafficking, biophysical properties and roles in physiological processes (Monyer et al., 1994; Flint et

al., 1997; Barria and Malinow, 2002; Lavezzari et al., 2004). Activation of NR2A-containing NMDAR promotes neuronal survival, while activation of NR2B-containing NMDAR increases neuronal death (Hardingham et al., 2002). Opposing roles of NR2A- and NR2B-containing NMDAR in induction of synaptic plasticity also have been reported (Liu et al., 2004), but these findings remain controversial because others showed a requirement for both NR2A- and NR2B-containing NMDAR (Tang et al., 1999; Barria and Malinow, 2005; Berberich et al., 2005; Weitlauf et al., 2005; Morishita et al., 2007). Lastly, chronic inhibition of NR2B-containing NMDAR dramatically reduced, while chronic inhibition of NR2A-containing NMDAR did not significantly affect subsequent electrographic seizures in organotypic hippocampal slice cultures (Wang and Bausch, 2004; Dong and Bausch, 2005). Effects of selective chronic inhibition of NR2A- and NR2B-containing NMDAR on excitatory and inhibitory circuits are unknown. Documenting these effects is critical given the status of NR2B-selective antagonists in clinical trials (Mony et al., 2009) and the need for a greater understanding of the contribution of NR2A- and NR2B-containing NMDAR in physiological processes.

In this study, we documented changes in $GABA_AR$-mediated neurotransmission because GABAergic transmission influences seizure expression and the effects of chronic NMDAR inhibition on GABAergic transmission are largely unknown. Based upon our previous findings, we hypothesized that chronic inhibition of NR2B-containing NMDAR would enhance, while chronic inhibition of NR2A-containing NMDAR would not dramatically affect $GABA_AR$-mediated transmission.

Materials and Methods

Choice of NMDAR antagonists. Representative NMDAR antagonists were selected based on their pharmacological properties as described previously (Wang and Bausch, 2004). The frequently used NR2B specific antagonist, Ro25,6981 (1 μM) was selected because of its high affinity and specificity for NR2B-containing NMDAR (Fischer et al., 1997; Zhao et al., 2005; Smith and McMahon, 2006; Zhou and Baudry, 2006). The NR2A-selective antagonist, NVP-AAM077 (50 nM) was chosen because it is often used in studies documenting the differential effects of NR2A- and NR2B-containing NMDAR (Massey et al., 2004; Zhou and Baudry, 2006) and its high preference for NR2A-containing NMDAR at this concentration (Auberson et al., 2002; Feng et al., 2004; Neyton and Paoletti, 2006). D(-)-2-amino-5-phosphonopentanoic acid (D-APV, 50 μM) was included to facilitate comparison with previous reports and was selected because of its specificity for NMDAR and frequent use in physiological and homeostatic plasticity studies.

Organotypic hippocampal slice cultures. Slice cultures were prepared using the interface method (Stoppini et al., 1991) as described previously (Bausch and McNamara, 2000; Bausch et al., 2006). All treatment of animals complied with National Institutes of Health, Department of Defense and institutional guidelines. Briefly, postnatal day 10-11 Sprague-Dawley rat pups (Taconic, Germantown, NY) were anesthetized with pentobarbital and decapitated. Brains were removed and cut into 400 μm transverse sections using a McIlwain tissue chopper. Hippocampal slices were separated from the entorhinal cortex in Gey's balanced salt solution (GBSS) composed of (in mM): 137 NaCl, 5 KCl, 0.25 $MgSO_4$, 1.5 $CaCl_2$, 1.05 $MgCl_2$, 0.84 Na_2HPO_4, 0.22 K_2HPO_4, 2.7

$NaHCO_3$, 41.6 glucose. The middle 4-6 slices of each hippocampus were placed onto tissue culture membrane inserts (Millipore, Bedford, MA) in a tissue culture dish containing medium. Medium consisted of 50% minimum essential medium, 25% Hank's buffered salt solution, 25% heat-inactivated horse serum, 0.5% GlutaMax, 10 mM HEPES (all from Invitrogen, Carlsbad, CA) and 6.5 mg/ml glucose (pH 7.2). Cultures were maintained at 37^{o}C under room air + 5% CO_2 and medium was changed three times per week. Cultures were treated with D(-)-2-amino-5-phosphonopentanoic acid (D-APV, 50 µM; Tocris Cookson, Ellisville, MO), Ro25,6981 hydrochloride (1 µM; Sigma), or NVP-AAM077 (50 nM; kind gift from Dr. Yves Auberson, Novartis Institutes for Biomedical Research, Basel, Switzerland) diluted in medium for the entire 17-21 DIV culture period. Vehicle-treated cultures were treated similarly, but drugs were omitted. Experiments were always conducted concurrently in cultures treated with vehicle and NMDAR antagonists under identical experimental conditions. Only cultures showing bright, well-defined cell layers were used for electrophysiological recordings.

Electrophysiological recordings. Recordings were conducted as described previously (Bausch et al., 2006) in dentate granule cells and interneurons at the dentate granule cell layer/hilus border (D/H border interneurons). Briefly, a small portion of the insert membrane containing a single cultured slice was cut and transferred to a submerged recording chamber mounted to a Zeiss Axioskop microscope with IR-DIC optics (Carl Zeiss Inc., Thornwood, NY). Slice cultures were continuously superfused (2-3 ml/min) with artificial cerebrospinal fluid (ACSF) composed of (in mM): 124 NaCl, 4.9 KCl, 1.2 KH_2PO_4 2.4 $MgSO_4$, 2.5 $CaCl_2$, 25.6 $NaHCO_3$, and 10 glucose equilibrated with 95% O_2, 5% CO_2. Tetrodotoxin (TTX, 1 µM; Sigma), D-APV (50 µM), bicuculline

methiodide (BMI, 10 μM; Tocris Cookson), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris Cookson), 4-aminopyridine (4-AP; Acros Organics), tetraethylammonium (TEA; Sigma), (2S)-[[(1S)-1-(3,4-Dichlorophenyl)ethyl] amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845 3 μM; Tocris Cookson), RS-α-cyclopropyl-4-phosphophenylglycine (CPPG, 200 μM; Tocris Cookson), dendrotoxin (200 nM; Sigma), dendrotoxin-K (100 nM; Sigma), margatoxin (10 nM; Sigma) and 10,10-bis(4-Pyridinylmethyl)-9(10*H*)-anthracenone dihydrochloride (XE 991, 10 μM; Tocris Cookson) were diluted immediately prior to use and acutely applied by bath superfusion. Recording pipettes were filled with (in mM): K-gluconate 125, KCl 13, HEPES 10, EGTA 10, MgATP 2 (pH 7.2 with KOH) for all whole-cell recordings. Data were collected using a Multiclamp 700A amplifier (2 kHz 8-pole Bessel filter), Digidata 320 A/D converter, and sampled at 10 kHz using pCLAMP software (all from Axon Instruments, Union City, CA).

All recordings were obtained from individual neurons in the suprapyramidal blade of the dentate gyrus / hilus border following $\geq$20 min. washout of NMDAR antagonists. Recordings were conducted at room temperature (RT) to minimize the likelihood of electrographic seizures during antagonist washout (Bausch and McNamara, 2000, 2004; Bausch et al., 2006). Data for action potential and membrane properties were collected using current-clamp recording conducted within 2 min. of establishing whole-cell configuration. The resting membrane potential (RMP) was documented using Multiclamp software. Input resistance (R_{in}) was calculated from the slope of the linear portion of a current-voltage plot of the change in membrane voltage in response to a series of 450 ms 25 pA steps using pClamp software. The first current step eliciting an action potential

was deemed the action potential threshold. The first action potential elicited at threshold was used to document action potential properties. Action potential rise and decay times were measured from 10%-100% and 100-10%, respectively; half-width was measured as the half-amplitude duration; fast afterhyperpolarization potential (fAHP) was measured from the baseline just prior to action potential rise to the peak of the hyperpolarized potential immediately followed the action potential (Faber and Sah, 2002).

For voltage-clamp recordings of synaptic currents, the membrane potential was clamped at -70 mV, and recordings were excluded if series resistance was >15 MΩ or varied more than 15% or the RMP was more positive than -50 mV for dentate granule cells and -40 mV for interneurons. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of D-APV (50 μM) and CNQX (10 μM). Miniature IPSCs (mIPSCs) were recorded after subsequent addition of TTX (1 μM). MiniAnalysis software (Synaptosoft Inc., Fort Lee, NJ) was used for analyses of all sIPSCs and mIPSCs. Detection threshold was set at 8 pA. Up to 80 synaptic currents from each cell were selected at a fixed sampling interval and compiled to generate cumulative probability plots (Suppl. Fig. 1).

For paired recordings between individual dentate granule cells and D/H border interneurons, the presynaptic neurons were current-clamped to generate action potentials and the postsynaptic neurons were voltage-clamped at a holding potential of -70 mV to record postsynaptic responses. An action potential was elicited in the presynaptic neuron using a brief 5 ms injection of depolarizing current, which was minimally sufficient to evoke a single action potential. To measure failure rate, 50 depolarizing stimuli were delivered at 0.3 Hz to the presynaptic neuron and subsequent synaptic currents were

recorded in the postsynaptic neurons. Failure rate was represented as the percentage of action potential-inducing stimuli that failed to evoke a postsynaptic response. To measure paired-pulse ratio, 2 stimuli were delivered to the presynaptic neuron with an interstimulus interval of 100 ms while synaptic currents were recorded in the postsynaptic neuron. Paired-pulse ratio was calculated as the amplitude of the second postsynaptic response normalized to the first.

Neurobiotin. Individual interneurons were filled with neurobiotin [0.4-0.5% (w/v) in the pipette solution; Vector, Burlingame, CA] during whole-cell recordings and visualized as described previously (Bausch et al., 2006) to identify the recorded neurons. Briefly, immediately after electrophysiological recordings, cultures were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), removed from the insert membrane, sunk in 30% sucrose in 0.1M PB containing 0.15 M NaCl and 2.7 mM KCl (PBS, pH 7.4) and stored at –70^{o}C. Thawed cultures were treated with 10% methanol and 0.6% H_2O_2 in PBS, blocked with 2% bovine serum albumin (BSA) and 0.75% Triton X-100 in PBS and incubated overnight at 4^{o}C in ABC elite (Vector) diluted according to kit instructions in PBS containing 2% BSA and 0.1% Triton X-100. Cultures were then treated with 0.05% 3,3'-diaminobenzidine (DAB, Sigma), 0.028% $CoCl_2$, 0.02% $NiSO_4 \cdot (NH_4)SO_4$ and 0.00075% H_2O_2 in PBS until staining was apparent. Cultures were mounted onto subbed glass slides, dehydrated, cleared and coverslipped. Representative interneurons were photographed and/or manually reconstructed using Neurolucida software (MicroBrightField Inc., Colchester, Vermont), a Zeiss Axioskop microscope equipped with 63X oil objective, MicroFire CCD camera (Optronics Inc., Goleta, CA), and motorized stage and focus encoder (Ludl Electronic Products Ltd.,

Exton, PA) as described previously (Bausch et al., 2006). Regions were defined as: molecular layer, supragranular regions of the dentate gyrus; granule cell layer, tightly packed layer of granule cells; hilus, region between the blades of the granule cell layer excluding the CA3c pyramidal cell layer; and CA3c, the pyramidal cell layers residing in the dentate gyrus. D/H border interneurons were classified based on axonal distribution and firing properties as shown in Fig. 2.

Statistics. Investigators were blinded to experimental groupings for all data analyses. Parametric data were represented as means $\pm$ SEM. Nonparametric data were represented as medians. Statistical analyses were performed with Sigma Stat software (SPSS Inc., Chicago, Illinois). Parametric data were tested for significance between experimental groups using an ANOVA with Holm-Sidak post hoc comparison (multiple groups), t-test (two groups) or paired t-test (before and after acute drug application in each chronic drug treatment group). Non-parametric data were tested for significance using ANOVA on Ranks. Significance was defined as $p \leq 0.05$. Cumulative probability distributions in Supplemental Figure 1 were tested for significance using a two-tailed Kolmogorov-Smirnov test using MiniAnalysis software; significance was defined as $p < 0.025$.

Results

The frequency of action potential-dependent sIPSCs, but not action potential-independent mIPSCs onto dentate granule cells was decreased following chronic inhibition of NR2B-containing NMDAR.

To begin to examine changes in GABAergic neurotransmission following chronic NMDAR inhibition, we first measured sIPSCs in individual granule cells using whole-cell voltage-clamp recordings. We found a dramatic (70 ± 7%) reduction in action potential-dependent sIPSC frequency following chronic inhibition of NR2B-containing NMDAR with Ro25,6981, but no significant changes following chronic NMDAR inhibition with the NR2A-prefering antagonist, NVP-AAM077 or the non-subunit selective NMDAR antagonist, D-APV (Fig. 1A1). Average sIPSC amplitude (Fig. 1A2) and action potential-independent mIPSC frequencies and amplitudes (Fig. 1B) in granule cells were not significantly altered following chronic NMDAR inhibition with any of the antagonists.

The frequency of action potential-dependent sIPSCs, but not action potential-independent mIPSCs onto dentate/hilar border interneurons was decreased following chronic inhibition of NR2B-containing NMDAR.

We next examined whether the effects of NMDAR antagonists on IPSCs were specific to dentate granule cells or also occurred in D/H border interneurons, which provide strong inhibition onto granule cells. An alteration in inhibition onto interneurons could affect presynaptic action potential-dependent GABA release by changing interneuron

excitability. D/H border interneurons fell into five distinct categories based upon axonal distribution and firing pattern (Fig. 2). Since no significant differences in the frequency or amplitude of sIPSCs or mIPSCs were apparent between different populations of D/H border interneurons, sIPSC and mIPSC data from all interneurons were compiled. Comparison of different treatment groups revealed a 66 ± 7% reduction in action potential-dependent sIPSC frequency in D/H border interneurons following chronic inhibition of NR2B-containing NMDAR with Ro25,6981, but no significant changes following chronic NMDAR inhibition with the NR2A-prefering antagonist, NVP-AAM077 or the non-subunit selective NMDAR antagonist, D-APV (Fig. 3A1). Spontaneous IPSC amplitude in D/H border interneurons was not significantly altered following chronic NMDAR inhibition with any of the antagonists, but a trend ($p=0.110$, ANOVA with Holm-Sidak posthoc comparison) toward a decrease was observed following chronic inhibition of NR2B-containing NMDAR with Ro25,6981 (Fig. 3A2). Action potential-independent mIPSC frequency and amplitude in D/H border interneurons were not significantly changed following chronic NMDAR inhibition (Fig. 3B). The virtually identical effects of chronic NMDAR inhibition on IPSCs in interneurons and granule cells suggest that reduced sIPSC frequency in granule cells was not caused by increased inhibition onto interneurons and that plasticity induced by chronic inhibition of NR2B-containing NMDAR in sIPSC frequency may occur in widespread subpopulations of interneurons.

Intrinsic active and passive membrane properties were not altered following chronic inhibition of NR2B-containing NMDAR.

The simplest and most obvious explanation for alterations in sIPSC but not mIPSC frequency is a change in interneuron action potential and/or intrinsic membrane properties. Therefore, we examined these properties in D/H border interneurons after treatment with NMDAR antagonists using whole-cell current-clamp recordings. Data were grouped by interneuron class because membrane and action potential properties were different in distinct interneuron populations (Table 1). Comparing the effects of chronic treatment with different NMDAR antagonists, we found a significantly more positive resting membrane potential (RMP) and increased action potential half-width, rise and decay times in axo-axonic cells following chronic treatment with D-APV and NVP-AAM077, respectively (Table 1). However, no other significant effects of chronic NMDAR inhibition on input resistance, resting membrane potential, afterhyperpolarization, or action potential threshold, amplitude, half-width, rise, decay or number were detected in D/H border interneurons (Table 1). These data suggest that altered somatic action potential and membrane properties were unlikely to account for the dramatic reduction in sIPSC frequency after chronic inhibition of NR2B-containing NMDAR.

Action potential-dependent release probability was unaltered following chronic inhibition of NR2B-containing NMDAR.

Fredj and Burrone (2009) recently demonstrated distinct vesicle pools for action potential-dependent and action potential-independent neurotransmitter release at CNS synapses. Reduced action potential-dependent release probability could decrease sIPSC frequency. Therefore, action potential-dependent release probability was documented

using paired recordings between D/H border interneurons and dentate granule cells. All IPSCs evoked in interneuron – granule cell pairs (eIPSCs) should be monosynaptic because inhibitory currents hyperpolarize rather than depolarize postsynaptic neurons and eIPSCs in granule cells displayed a single, short latency distribution (1.89 ± 0.07 ms; range, 0.5-4.9 ms). Evoked IPSC failure data were grouped into high-frequency (basket and axo-axonic cells) and adapting (HIPP, HICAP and everywhere cells) firing interneurons because the eIPSC failure rate was different across but not within these populations (Maccaferri et al., 2000; Xiang et al., 2002). Chronic treatment with NMDAR antagonists had no significant effect on eIPSC failure rate in either high-frequency (Fig. 4B) or adapting firing (Fig. 4C) interneurons. Since the failure rate measures a combination of release probability and axon conduction failure, we further investigated alterations in release probability using paired-pulse stimulation of presynaptic interneurons and recordings of subsequent paired-pulse IPSC ratios in granule cells. Paired stimuli with an interstimulus interval of 100 ms were used to document release probability without the confound of high frequency action potential-induced conduction failure (Debanne, 2004). Paired-pulse ratios were not significantly different in distinct populations of D/H border interneurons, so data from all interneuron - granule cell pairs were compiled. Chronic treatment with NMDAR antagonists did not significantly affect paired-pulse eIPSC ratios (Fig. 5B), suggesting no change in action potential-dependent GABA release probability.

Lastly, changes in presynaptic $GABA_B$ and group III metabotropic glutamate (mGlu) receptor function could alter action potential-dependent release probability and reduce sIPSC frequency (Olpe et al., 1982; Niswender et al., 2008). However, this possibility is

unlikely since acute pharmacological blockade of $GABA_B$ receptors and group III mGlu receptors with CGP55845 (3 μM) and CPPG (200 μM), respectively, had no impact on sIPSC frequency or amplitude in granule cells from either vehicle- or Ro25,6981-treated cultures (Fig. 6). Taken together, these data suggest that alterations in action potential-dependent release probability could not account for reduced sIPSC frequency following chronic inhibition of NR2B-containing NMDAR.

Acute blockade of Kv channels with 4-AP or TEA occluded the effect of chronic inhibition of NR2B-containing NMDAR on sIPSC frequency.

Acute pharmacological blockade or genetic deletion of voltage-gated potassium (Kv) channels can increase action potential-dependent GABA release (Southan and Robertson, 1998; Zhang et al., 1999; Cunningham and Jones, 2001; Goldberg et al., 2005) by modulating neuronal membrane potential and/or action potential threshold, duration and firing frequency. Although we found no significant alterations in somatic membrane or action potential properties, changes in axonal properties could reduce sIPSC frequency by altering axonal excitability (Meir et al., 1999; Debanne, 2004; Dodson and Forsythe, 2004) and would not be detected using whole-cell recordings. Therefore, we first tested whether two broad Kv channel blockers, 4-aminopyridine (4-AP) and tetraethylammonium (TEA) applied acutely during recordings of sIPSCs, but after chronic NMDAR inhibition, could occlude the dramatic reduction in sIPSC frequency observed following chronic inhibition of NR2B-containing NMDAR. Concentration-response experiments were conducted because the potency of these blockers can provide clues as to the identity of altered Kv channels. Concentration-response analyses revealed

that as little as 10 μM 4-AP or 20 mM TEA was sufficient to occlude the effects of chronic Ro25,6981 on sIPSC frequency in granule cells (Fig. 7). Based on previously reported potencies for recombinant Kv channels (Mathie et al., 1998; Judge and Bever, 2006), no Kv channel subtype strictly fits our data. However, native endogenous Kv channels are not likely to be homotetramers, but rather heterotetramer assemblies containing a variety of Kv1 subunits. Pharmacology, biophysical properties, expression, trafficking and localization of Kv1 channels are influenced by the coassembly of heteromeric Kv1 subunits, presence of β subunits, composition of membrane lipids, glycosylation, phosphorylation as well as the expression system and amount mRNA injected into oocytes (Harvey, 1997; Robertson, 1997; Mathie et al., 1998; Oliver et al., 2004; Trimmer and Rhodes, 2004-reviews). That said, the occlusion of chronic Ro25,6981-mediated effects by 10 μM 4-AP in our experiments suggested the involvement of Kv3 (Grissmer et al., 1994; Lien et al., 2002). Arguing against this possibility, Kv3 channels modulate action potential duration and are expressed predominantly in parvalbumin-containing axo-axonic and basket cells (Gan and Kaczmarek, 1998; Rudy and McBain, 2001) and we found no significant changes in action potential decay in axo-axonic or basket cells following chronic Ro25,6981 treatment. Moreover, 0.2 mM TEA, which selectively blocks Kv3 channels (Aiyar et al., 1994) did not significantly affect sIPSCs following chronic Ro25,6981 treatment. Occlusion of chronic Ro25,6981-mediated decreases in sIPSC frequency by 20 mM TEA implicated increased Kv1.1, Kv1.2, Kv1.3 and/or Kv2.1 channel function (Mathie et al., 1998; Judge and Bever, 2006). Arguing against altered Kv2.1 channel function, Kv2.1 channels are predominantly expressed in somata and play a critical role in regulating

action potential and membrane properties (Misonou et al., 2005), but we found no significant change in somatic action potential or membrane properties in interneurons following chronic Ro25,6981 treatment. Therefore, we concentrated on potential alterations in Kv1 function because Kv1 channels are localized predominantly to axons and are implicated in the regulation of presynaptic neurotransmitter release (Southan and Robertson, 1998; Zhang et al., 1999; Lai and Jan, 2006).

Enhanced Kv1 channel function contributed to reduced sIPSC frequency following chronic inhibition of NR2B-containing NMDAR.

To test the possibility that alterations in Kv1 channel function contributed to chronic Ro25,6981-mediated decreases in sIPSC frequency, we first acutely applied relatively broad Kv1 channel blockers. Acute blockade of Kv1.1, Kv1.2 and Kv1.6 with α-dendrotoxin (200 nM; Grissmer et al., 1994; Lambe and Aghajanian, 2001) (Fig. 8A) or Kv1.1, Kv1.3 and Kv1.6 with agitoxin (30 nM; Garcia et al., 1994; Southan and Robertson, 1998) (not shown) dramatically increased sIPSC frequency in granule cells from Ro25,6981-treated cultures, and more importantly, occluded chronic Ro25,6981-induced effects on sIPSC frequency. We next utilized more specific Kv blockers to more precisely document the involvement of Kv1 channel subtypes. Acute blockade of Kv1.3-containing channels with margatoxin (10 nM; Southan and Robertson, 2000; Akhtar et al., 2002) also completely occluded chronic Ro25,6981-mediated decreases in sIPSC frequency (Fig. 8B). However, acute blockade of Kv1.1-containing Kv1 channels with dendrotoxin-K (100 nM; Vicente et al., 2006) increased sIPSC frequency and amplitude, but did not occlude chronic Ro25,6981-mediated decreases in sIPSC frequency (Fig. 8C).

Another Kv channel, Kv7 is expressed in hippocampal and dentate gyrus interneurons, can co-localize with Kv1 channels in axonal initial segments, juxtaparanodal regions and synaptic terminals and can modulate inter-spike interval and GABA release (Cooper et al., 2001; Devaux et al., 2004; Martire et al., 2004; Trimmer and Rhodes, 2004; Lai and Jan, 2006; Lawrence et al., 2006). Therefore, a specific Kv7 channel blocker was used as a control to show specificity of Ro25,6981-mediated effects on Kv1 channel function. In contrast to Kv1 channel blockers, acute pharmacological blockade of Kv7 with XE 991 (10 μM) did not significantly affect sIPSC frequency or amplitude in granule cells and did not occlude chronic Ro25,6981-mediated effects on sIPSC frequency (Fig. 8D). The negative outcome with XE 911 is consistent with the findings of Martire et al. (2004), who showed that 10 μM XE 991 blocked retigabine-mediated increases in high K^+-induced neurotransmitter release from synaptosomes, but had no effect alone. Taken together, our findings imply that up-regulated Kv1 channel function reduced sIPSC frequency following chronic inhibition of NR2B-containing NMDAR with Ro25,6981 and that these channels contain Kv1.3, Kv1.6 and possibly Kv1.2. Since Kvα1.3 and Kvα1.6 can form a tetramer with Kvα1.2/1.4 and Kvβ2 (Shamotienko et al., 1997), the Kv1 channels underlying chronic Ro25,6981-mediated effects on sIPSC frequency are likely to be heteromeric complexes.

Discussion

Spontaneous IPSC frequency was reduced dramatically in both dentate granule cells and interneurons following chronic inhibition of NR2B-containing NMDAR with Ro25,6981, but mean sIPSC amplitude, mIPSC frequency and mIPSC amplitude were not significantly altered, suggesting diminished action potential-dependent GABA release. Chronic treatment with NVP-AAM077 or D-APV had no significant effect on any of these measures. Reduced sIPSC frequency was not due to down-regulated $GABA_AR$ because mIPSC amplitude was unchanged. Decreased excitatory or increased inhibitory drive to interneurons did not reduce sIPSC frequency because fast excitatory transmission was blocked and sIPSC frequency in interneurons was reduced. Reduced sIPSC frequency did not arise from altered somatic interneuron membrane/action potential properties because these properties were unaltered. Altered action potential-dependent release probability and mGluR/$GABA_B$ receptor modulation of GABA release cannot account for reduced sIPSC frequency because paired granule cell/interneuron recordings revealed no significant change in eIPSC failure rate or paired-pulse ratio, and mGluR and $GABA_B$ receptor inhibitors did not affect sIPSC frequency. However, chronic Ro25,6981-mediated reductions in sIPSC frequency were occluded by dendrotoxin, margatoxin, agitoxin, but not dendrotoxin-K or XE991, suggesting that increased Kv1 channel function contributes to diminished action potential-dependent GABA release following chronic inhibition of NR2B-containing NMDAR with Ro25,6981 and that these Kv1 channels may be heteromeric complexes containing Kv1.3, Kv1.6 and possibly Kv1.2. Our conclusions are consistent with the predominant NR2B (Telfeian et al., 2003) and Kv1.6 expression in hippocampal interneurons and Kv1.2, 1.6, β1 in dentate

interneurons (Rhodes et al., 1997) as well as presynaptic Kv1.3 modulation of GABA release in hippocampus (Ohno-Shosaku et al., 1996). To our knowledge, this is the first report showing dramatically reduced action potential-dependent GABA release and the involvement of increased Kv1 channel function following chronic inhibition of NR2B-containing NMDAR.

Potential localization of increased Kv1 channel function responsible for decreased sIPSC frequency following chronic inhibition of NR2B-containing NMDAR.

Potassium channels regulate neuronal excitability, axonal action potential propagation and neurotransmitter release. Accordingly, Kv1 channels are localized predominantly to axons, but also reside in somata, dendrites, (Trimmer and Rhodes, 2004; Lai and Jan, 2006; Arnold, 2007) and glia (Schlichter et al., 1996; Smart et al., 1997; Hallows and Tempel, 1998). Astrocytes modulate inhibitory neurotransmission (Kang et al., 1998; Yamazaki et al., 2005) and express Kv1.3, Kv1.6 (Smart et al., 1997; Lee et al., 2009) and NR2B-containing NMDAR (Conti et al., 1996). However, dendrotoxin is specific for neuronal Kv1 channels (Grissmer et al., 1994; Lambe and Aghajanian, 2001), suggesting that neuronal up-regulation of Kv1 channel function contributed to reduced sIPSC frequency. Likewise, altered somatodendritic Kv1 channels in interneurons were unlikely to reduce sIPSC frequency because mIPSC amplitude and somatic action potential/membrane properties were unaltered. Thus, the Kv1 channels responsible for reduced sIPSC frequency following chronic Ro25,6981 treatment are likely to reside in interneuron axons and/or synaptic terminals.

Axonal potassium channels control synaptic efficacy by influencing action potential invasion into nerve terminals (Lambe and Aghajanian, 2001) and reside in axonal initial segments (AIS), branch points, axonal swellings, juxtaparanodal regions, synaptic terminals, and pre-terminal axonal "necks" (Sheng et al., 1993; Rhodes et al., 1997; Cooper et al., 1998; Zhang et al., 1999; Geiger and Jonas, 2000; Inda et al., 2006; Van Wart et al., 2007). Kv1 channels in the AIS dampen near-threshold excitability in fast-spiking interneurons (Goldberg et al., 2008). Therefore, up-regulated AIS Kv1 channel function could lead to reduced sIPSC frequency. Kv1 channels in branch points and axonal swellings influence conduction fidelity (Krnjevic and Miledi, 1959; Grossman et al., 1979) and Kv1 channels in synaptic terminals and pre-terminal necks modulate spike duration, terminal excitability, neurotransmitter release probability and subsequent PSC amplitude (Geiger and Jonas, 2000). However, we detected no significant changes in IPSC amplitude, failures or paired-pulse ratio in paired recordings, arguing against Kv1-mediated changes in branch point or synaptic terminal excitability. Lastly, deletion of Kv1.1 at septate-like junctions increases the probability of cross excitation of closely apposed axons (Chiu et al., 1999). By analogy then, increased Kv1 function at septate-like junctions may decrease cross activation of closely apposed axons and decrease sIPSC frequency without altering conduction failures or release probability in paired recordings between single interneurons and granule cells. Taken together, the most likely locations for up-regulated Kv1 channel function following chronic inhibition of NR2B-containing NMDAR are neuronal AIS and/or axonal septate-like junctions.

Potential mechanisms underlying increased Kv1 channel function following chronic inhibition of NR2B-containing NMDAR.

Previous studies showed that NMDAR activation alters somatodendritic membrane surface expression and voltage-dependent channel inactivation of Kv4.2 (Kim et al., 2007; Lei et al., 2008) and changes phosphorylation, somatic surface expression, lateral diffusion, and voltage-dependent channel activation of Kv2.1 (Misonou et al., 2004; Yao et al., 2009). Increased local intracellular calcium elicited directly by NMDAR activation and indirectly via membrane depolarization and subsequent voltage-gated Ca^{2+} channel activation is thought to underlie modulation of Kv channel function. NMDAR modulation of somatodendritic Kv channels is thought to depend on a close spatial relationship between transient local NMDAR-mediated Ca^{2+} influx (Mainen et al., 1999; Sabatini et al., 2002) and Kv channels (Smart et al., 1997; Seifert and Steinhauser, 2001; Engelman and MacDermott, 2004; Trimmer and Rhodes, 2004). However, in our study chronic inhibition of NR2B-containing NMDA most likely altered axonal Kv1 channel function, raising the question of how chronic NMDAR inhibition elicited alterations in axonal Kv channels. Recently, Fiszman and colleagues (2005) as well as Christie and Jahr (2008) reported that currents mediated by somatodendritic NMDAR are transmitted to cerebellar basket cell terminals via axonal electrotonic current propagation. These findings suggest that a spatial close relationship between NMDAR and Kv1 channels is not necessary for NMDAR-mediated Kv1 channel regulation and provide support for NMDAR modulation of axonal Kv1 channels. Additionally, presynaptic NR2B-containing NMDAR exists in a variety of neuronal subtypes (Berretta and Jones, 1996; Sjostrom et al., 2003; Yang et al., 2006; Brasier and Feldman, 2008), including cerebellar

interneurons (Glitsch and Marty, 1999; Duguid and Smart, 2004; Huang and Bordey, 2004; Fiszman et al., 2005; but see Christie and Jahr, 2008) and resides primarily in extrasynaptic axons (Gracy and Pickel, 1995; Gracy et al., 1997). Although similar findings have yet to be reported in hippocampal or dentate interneurons, these data provide possible scenarios by which NMDAR activation and by analogy blockade of dendritic and/or axonal NR2B-containing NMDAR could modulate axonal Kv channel function.

The high NR2B expression in hippocampal interneurons (Telfeian et al., 2003) and unique biophysical properties of NR2B-containing NMDAR may account for the specific effects of chronic NR2B-containing NMDAR inhibition on Kv channel function. NR2B conveys a higher Ca^{2+} permeability than NR2A in heteromeric NMDAR and preferentially forms complexes with downstream Ca^{2+}-binding proteins such as CaMKII (Leonard et al., 1999; Strack et al., 2000) and RasGRF1 (Krapivinsky et al., 2003). Since elevated NMDAR-mediated Ca^{2+} influx reduces Kv channel membrane surface localization (Lei et al., 2008), chronic NR2B-containing NMDAR inhibition may increase Kv channel surface expression via diminished Ca^{2+} entry. Additionally, NMDAR activation increases Kv1 channel phosphorylation (Tao et al., 2005), which modulates channel conductance, open probability and voltage sensitivity (Vogalis et al., 1995; Peretz et al., 1996; Kwak et al., 1999b; Kwak et al., 1999a), suggesting that altered Kv1 channel phosphorylation also may increase Kv1 channel function (Tao et al., 2005).

Potential role of altered GABA transmission and Kv1 channel function in seizure expression.

Together with results from our previous studies (Wang and Bausch, 2004; Dong and Bausch, 2005), we showed that reduced action potential-dependant GABA release onto dentate granule cells and interneurons is associated with enhanced Kv1 function and reduced seizure expression. Moreover, increased GABA release with no change in glutamatergic transmission in cerebral cortex (van Brederode et al., 2001) has been associated with spontaneous seizures in Kv1.1 null mice (Smart et al., 1998; Rho et al., 1999; Brew et al., 2007). Although the Kv1 channels underlying the changes in GABAergic inhibition are different, both groups of findings are at odds with data showing that therapeutic enhancement of GABAergic transmission can control seizures and blockade of $GABA_AR$-mediated transmission is seizurogenic (Burt, 1962; Paul et al., 1979). This discrepancy suggests either that the described changes in GABAergic transmission do not contribute to the epileptic phenotype or that a simple imbalance between excitatory and inhibitory transmission do not completely account for changes in seizure genesis.

In the adult brain GABAergic transmission causes hyperpolarization via Cl^- influx and shunting inhibition due to increased membrane permeability (Mann and Paulsen, 2007). Since the Cl^- reversal potential is close to the resting membrane potential in mature dentate granule cells (Staley and Mody, 1992; Scharfman, 1994; Williamson et al., 1995) and cortical pyramidal cells (Gulledge and Stuart, 2003), shunting inhibition predominates. Recently, Vida et al. (2006) reported that shunting inhibition primarily synchronizes neuronal firing rather than balancing excitation in the dentate gyrus.

Therefore, diminished action potential-dependent GABAergic transmission may reduce synchronization of neuronal firing and subsequent seizure expression.

Table 1. Membrane Properties in hilar Interneurons

Treatment	R_{IN} (MΩ)	RMP (mV)	Action Potential								
			Threshold (mV)	Amplitude (mV)	Half Width (ms)	Rise Time (ms)	Decay Time (ms)	fAHP (mV)	# at Threshold	# at 200 pA	# at -30 mV
Axo-axonic cell											
Vehicle	70 ± 4 (15)	-58.1 ± 0.9 (14)	-35.0 ± 0.5 (15)	69 ± 2 (15)	0.92 ± 0.04 (15)	0.36 ± 0.01 (15)	0.96 ± 0.05 (15)	-13.7 ± 0.6 (15)	2.0 (15)	1.0 (15)	9.0 (31)
D-APV	79 ± 4 (5)	-52.4 ± 0.9 (5)[a, c, d]	-32.7 ± 1.0 (5)	63 ± 3 (5)	0.97 ± 0.10 (5)	0.46 ± 0.04 (5)	0.92 ± 0.10 (5)	-13.9 ± 1.7 (5)	2.0 (5)	1.0 (5)	9.0 (26)
Ro25,6981	82 ± 7 (10)	-56.6 ± 0.4 (10)	-33.3 ± 0.9 (10)	64 ± 3 (10)	0.93 ± 0.07 (10)	0.41 ± 0.03 (10)	0.89 ± 0.09 (10)	-14.4 ± 0.6 (10)	2.0 (10)	5.0 (9)	7.0 (28)
NVP-AAM077	86 ± 9 (8)	-56.4 ± 0.9 (9)	-34.2 ± 1.1 (8)	65 ± 3 (8)	1.38 ± 0.14 (8)[a, b, c]	0.53 ± 0.03 (8)[a, c]	1.53 ± 0.23 (8)[a, b, c]	-13.5 ± 1.0 (8)	2.0 (8)	0.5 (8)	9.0 (31)
Basket cell											
Vehicle	58 ± 4 (7)	-56.5 ± 0.6 (7)	-33.3 ± 0.9 (7)	64 ± 2 (7)	1.03 ± 0.04 (7)	0.41 ± 0.01 (7)	1.05 ± 0.06 (7)	-15.1 ± 0.9 (7)	2.0 (7)	1.0 (6)	13.0 (5)
D-APV	77 ± 4 (10)	-56.0 ± 0.7 (10)	-31.4 ± 0.9 (10)	61 ± 2 (10)	1.00 ± 0.05 (10)	0.46 ± 0.03 (10)	0.97 ± 0.06 (10)	-16.4 ± 0.7 (10)	2.5 (10)	0.0 (9)	8.0 (7)
Ro25,6981	72 ± 6 (9)	-56.4 ± 0.4 (9)	-30.7 ± 1.0 (9)	58 ± 2 (9)	0.92 ± 0.07 (9)	0.44 ± 0.03 (9)	0.83 ± 0.07 (9)	-15.6 ± 0.6 (9)	2.0 (9)	0.0 (7)	9.5 (8)
NVP-AAM077	62 (1)	-58.7 (1)	-38.3 (1)	76 (1)	0.56 (1)	0.31 (1)	0.46 (1)	-10.1 (1)	2.0 (1)	3.0 (1)	
HICAP cell											
Vehicle	248 (2)	-53.7 (2)	-33.0 (2)	54 (2)	3.60 (2)	1.09 (2)	3.70 (2)	-8.4 (2)	1.0 (2)	6.5 (2)	3.0 (1)
D-APV	190 ± 91 (3)	-51.7 ± 2.5 (3)	-33.8 ± 0.5 (3)	58 ± 3 (3)	2.17 ± 0.59 (3)	0.67 ± 0.19 (3)	2.40 ± 0.57 (3)	-11.2 ± 2.2 (3)	3.0 (3)	11.0 (3)	10.0 (3)
Ro25,6981	187 ± 54 (6)	-54.4 ± 1.5 (6)	-33.8 ± 0.8 (6)	65 ± 3 (6)	1.80 ± 0.21 (6)	0.71 ± 0.07 (6)	2.00 ± 0.28 (6)	-12.0 ± 1.4 (6)	2.0 (6)	3.5 (6)	9.0 (6)
NVP-AAM077	237 ± 29 (3)	-51.3 ± 0.5 (4)	-34.9 ± 0.9 (3)	73 ± 3 (3)	1.70 ± 0.24 (3)	0.58 ± 0.02 (3)	1.86 ± 0.20 (3)	-13.0 ± 1.39 (3)	2.0 (3)	11.0 (3)	11.0 (3)
HIPP cell											
Vehicle	166 ± 35 (6)	-54.5 ± 1.7 (8)	-34.6 ± 1.2 (6)	64 ± 2 (6)	1.66 ± 0.19 (6)	0.63 ± 0.08 (6)	1.63 ± 0.20 (6)	-13.6 ± 1.8 (6)	2.0 (6)	1.0 (18)	7.0 (5)
D-APV	234 ± 47 (6)	-50.9 ± 1.1 (7)	-33.6 ± 0.8 (6)	64 ± 3 (6)	1.99 ± 0.27 (6)	0.66 ± 0.06 (6)	2.18 ± 0.24 (6)	-11.2 ± 1.4 (6)	2.0 (6)	1.0 (14)	10.0 (6)
Ro25,6981	276 ± 47 (11)	-53.2 ± 1.2 (13)	-33.9 ± 1.0 (11)	61 ± 4 (11)	2.56 ± 0.29 (11)	0.87 ± 0.09 (11)	2.71 ± 0.30 (11)	-11.9 ± 1.1 (11)	2.0 (11)	1.0 (17)	7.0 (11)
NVP-AAM077	248 ± 42 (5)	-51.4 ± 1.3 (7)	-32.8 ± 1.7 (5)	63 ± 5 (5)	2.06 ± 0.21 (5)	0.81 ± 0.14 (5)	2.22 ± 0.38 (5)	-13.3 ± 2.4 (5)	2.0 (5)	0.0 (9)	8.0 (5)
Everywhere cell											
Vehicle	254 ± 22 (33)	-51.5 ± 0.6 (37)	-35.4 ± 0.4 (34)	65 ± 1 (34)	2.33 ± 0.12 (33)	0.78 ± 0.03 (33)	2.62 ± 0.14 (34)	-11.1 ± 0.5 (34)	1.0 (34)	9.0 (34)	9.0 (31)
D-APV	244 ± 26 (25)	-50.8 ± 0.6 (29)	-35.2 ± 0.4 (26)	65 ± 1 (26)	2.29 ± 0.17 (25)	0.72 ± 0.04 (25)	2.57 ± 0.18 (26)	-11.0 ± 0.7 (26)	3.0 (26)	10.0 (26)	9.0 (26)
Ro25,6981	266 ± 17 (28)	-52.2 ± 0.6 (31)	-34.6 ± 0.5 (28)	62 ± 2 (28)	2.47 ± 0.14 (28)	0.83 ± 0.05 (28)	2.64 ± 0.17 (28)	-11.1 ± 0.7 (28)	2.0 (28)	9.0 (28)	7.0 (28)
NVP-AAM077	295 ± 23 (33)	-53.1 ± 0.4 (35)	-35.2 ± 0.5 (37)	65 ± 2 (33)	2.26 ± 0.10 (33)	0.76 ± 0.03 (33)	2.49 ± 0.12 (33)	-11.6 ± 0.5 (33)	2.0 (33)	9.0 (33)	9.0 (31)

Means ± SEM, except action potential numbers, which indicates medians. *Abbreviations*: R_{IN}, input resistance; RMP, resting membrane potential; fAHP, fast after hyperpolarization potential. HIPP cell, hilar perforant pathway-related cell. HICAP cell, hilar commissural-associational pathway-associated cell. The number of granule cells / hippocampal slice cultures is indicated in parentheses. [a], different than vehicle; [b], different than D-APV; [c], different than Ro25,6981; [d], different than NVP-AAM077; $p<0.05$, ANOVA with Holm-Sidak *post hoc* comparison.

Figure 1. sIPSCs in granule cell

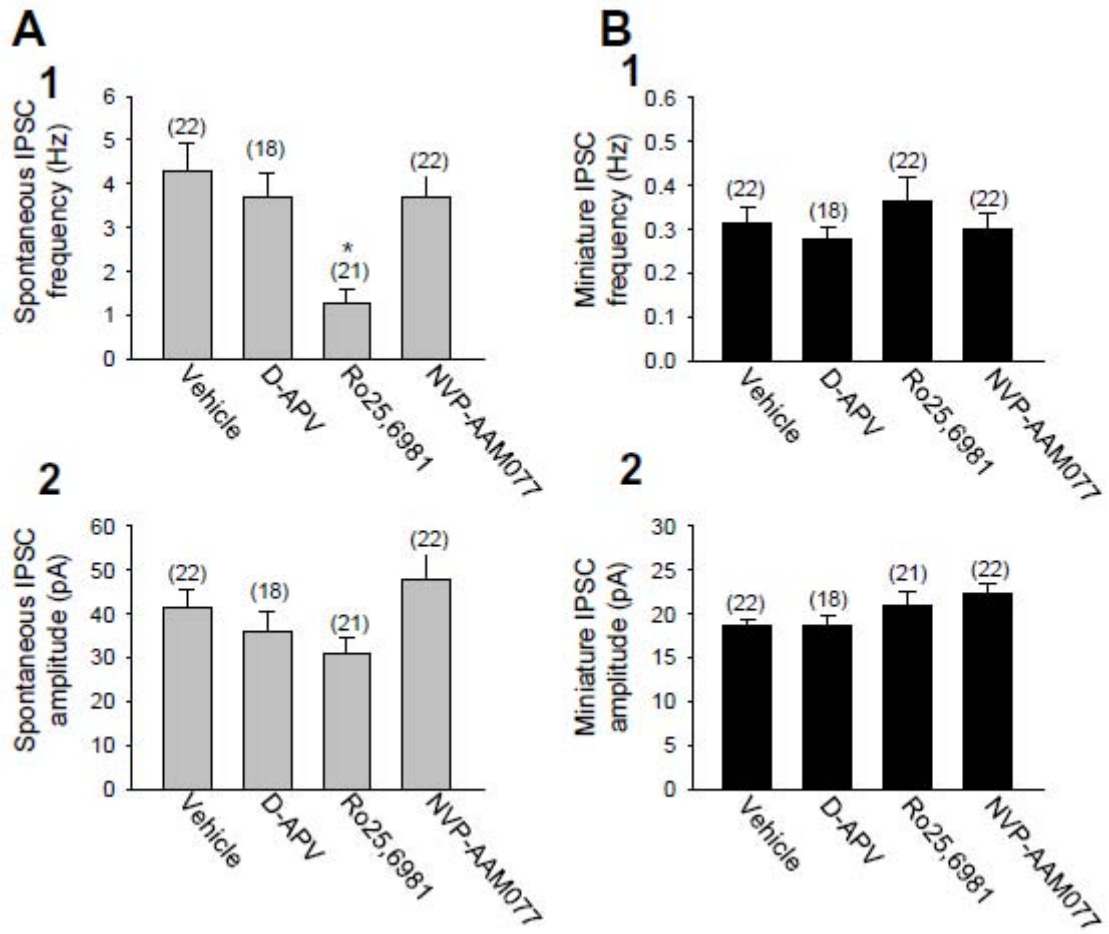


Figure 1. Spontaneous IPSC frequency was dramatically reduced in granule cells from hippocampal slice cultures treated chronically with Ro25,6981. Spontaneous IPSCs were recorded at a -70 mV holding potential in recording buffer containing D-APV (50 μM) and CNQX (10 μM). Miniature IPSCs were recorded after subsequent addition of TTX (1 μM). Bar graphs reveal that ***A,*** sIPSC ***A1,*** frequency was dramatically reduced but ***A2,*** amplitude was not significantly altered in granule cells following chronic treatment with Ro25,6981. ***B,*** Miniature IPSC ***B1,*** frequency and ***B2,*** amplitude in granule cells were not significantly altered following chronic treatment with NMDAR antagonists. The number of granule cells / slice cultures is indicated in parentheses. *, different than vehicle, D-APV and NVP-AAM077; $p<0.05$, ANOVA with Holm-Sidak *post hoc* comparison.

Figure 2. Interneuron morphology and firing patterns

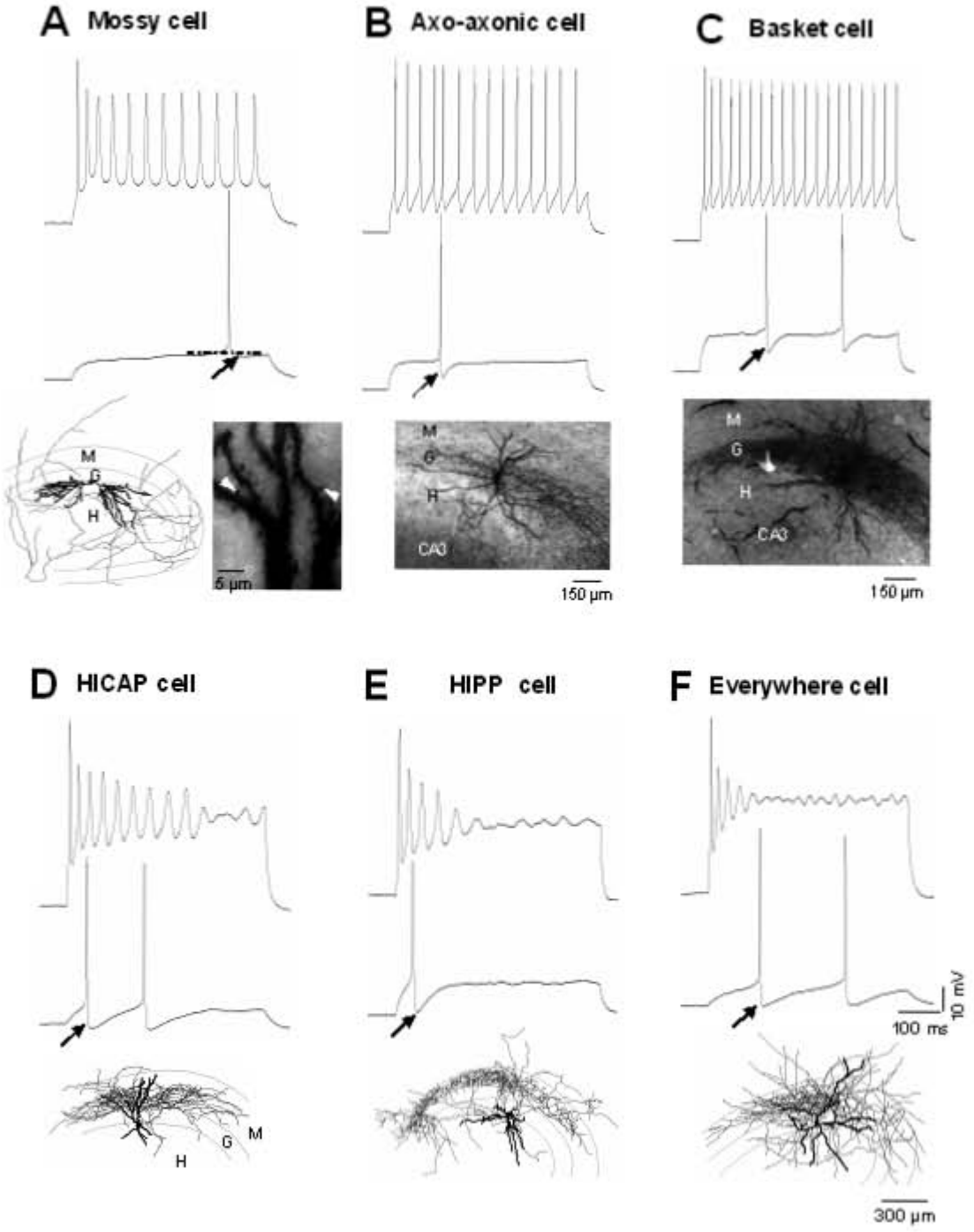


Figure 2. Firing patterns and axonal distributions were used to classify different types of interneurons. Interneurons were characterized as described previously (Halasy and Somogyi, 1993; Han et al., 1993; Buckmaster and Schwartzkroin, 1995a,b; Mott et al., 1997) except everywhere cells, which were described by Mott et al. (1997) but named here. ***A-E top,*** show representative traces from the first current step to elicit strong adaptation and spike broadening in HICAP, HIPP and everywhere cells, and a corresponding current step amplitude for mossy, axo-axonic and basket cells. ***A-E middle,*** show representative traces from the first current step to elicit an action potential. ***A-E bottom,*** show representative morphology and axonal distributions of neurobiotin- filled neurons. ***A,*** Mossy cells were characterized by high-frequency firing, a shallow AHP (dashed line, plateau potential), dendrites covered with dense "thorny excrescences" (bottom right, white arrowheads), and axons distributed throughout the dentate gyrus and hilus. Recorded cells with these characteristics were excluded from further analyses. ***B,*** Axo-axonic cells were characterized by high-frequency firing, action potentials with deep, short duration AHP, as well as large somata, chandelier-like rows of boutons and axonal arborizations predominantly in the granule cell and the CA3c pyramidal cell layers. ***C,*** Basket cells displayed high-frequency firing, action potentials with deep, short duration AHP, large somata and axons almost entirely restricted to the granule cell layer with net-like boutons surrounding granule cells. ***D,*** Hilar commissural-associational pathway associated interneurons (HICAP cells) were defined by adapting firing, deep, long-lasting AHP, and axonal collaterals distributed predominantly in the outer granule cell layer and the inner 1/3 of the molecular layer. Dendrites usually bifurcated bidirectionally into the molecular layer after crossing the granule cell layer and the hilus, and were of either aspiny or sparsely spiny. ***E,*** Hilar perforant pathway-related interneurons (HIPP cells) were characterized by adapting firing, deep intermediate-lasting AHP, and axonal collaterals distributed predominantly in the outer 2/3 of the molecular layer. Dendrites were often restricted to the hilus and covered with long thin spines. ***F,*** Everywhere cells were characterized by adapting firing, deep long duration AHP and axons that arborized radially throughout all regions of the dentate gyrus, hilus and the CA3 pyramidal cell layer. Representative mossy, axo-axonic, basket, HICAP, HIPP and everywhere cells were taken from Ro25,6981-, Ro25,6981-, vehicle-, D-APV-, memantine- and NVP-

AAM077-treated cultures, respectively. Arrows in A-F middle indicate AHP. Scale bars in F, middle apply to all electrophysiological traces; scale bar in F, bottom applies to all digitally reconstructed neurons in A, D-F. *Abbreviations:* m, molecular layer; g, granule cell layer; h, hilus; HICAP cell, hilar commissural-associational pathway-associated interneuron; HIPP cell, hilar perforant pathway-associated interneuron; EC, everywhere cell; BC, basket cell. Thick black lines in all digitally reconstructed neurons denote dendrites; thin black lines, axons; thin gray lines delineate regions.

Figure 3. sIPSCs in Interneuron

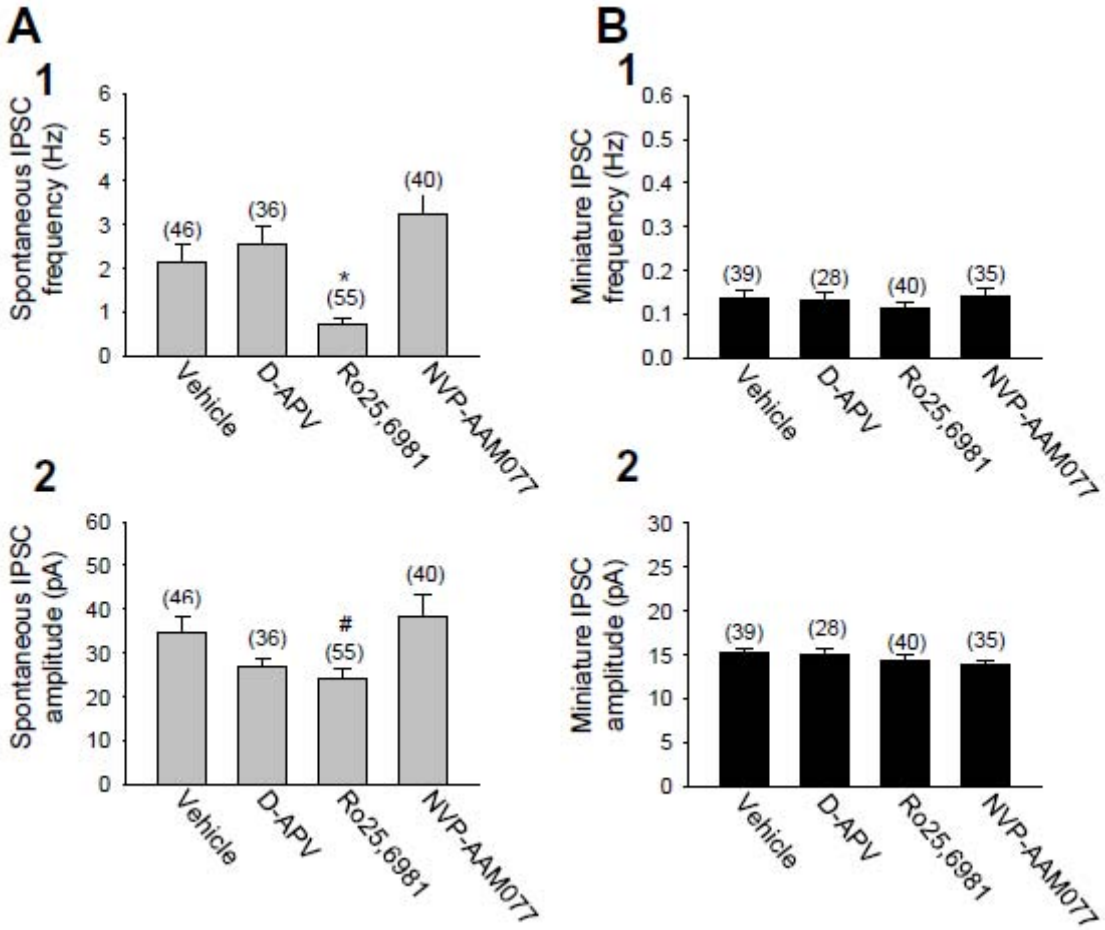


Figure 3. Spontaneous IPSC frequency was dramatically reduced in dentate/hilar border interneurons from hippocampal slice cultures treated chronically with Ro25,6981. Spontaneous IPSCs were recorded at a -70 mV holding potential in recording buffer containing D-APV (50 μM) and CNQX (10 μM). Miniature IPSCs were recorded after subsequent addition of TTX (1 μM). Data from all interneurons were compiled because no significant differences between different populations of D/H border interneurons were apparent for sIPSC and mIPSC. Bar graphs reveal that compared to vehicle ***A,*** sIPSC ***A1,*** frequency was dramatically reduced but ***A2,*** amplitude was not significantly altered in interneurons following chronic treatment with Ro25,6981. ***B,*** Miniature IPSC ***B1,*** frequency and ***B2,*** amplitude in interneurons were not significantly altered following chronic treatment with NMDAR antagonists. The number of interneurons / slice cultures is indicated in parentheses. *, different than vehicle, D-APV and NVP-AAM077; #, different than NVP-AAM077; $p<0.05$, ANOVA with Holm-Sidak *post hoc* comparison.

Figure 4. Failure rate of eIPSCs

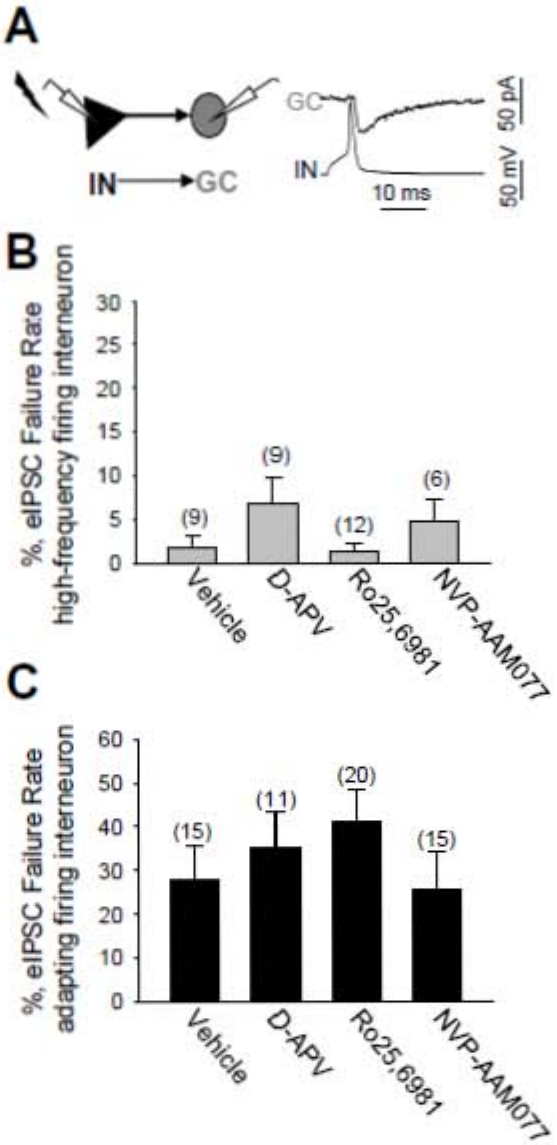


Figure 4. The failure rate of evoked IPSCs (eIPSCs) in granule cells was not significantly altered following chronic NMDAR inhibition. Paired whole-cell recordings between single dentate granule cells and D/H border interneurons were conducted to document the failure rate of eIPSCs. Presynaptic interneurons were current-clamped at their resting membrane potential and a brief 5 ms depolarizing current step, which was minimal for eliciting a single action potential, was applied 50 times at a frequency of 0.3 Hz. Evoked IPSCs were recorded in the postsynaptic granule cell, which was voltage-clamped at -70 mV. Failure data were grouped into high-frequency (basket and axo-axonic cells) and adapting (HIPP, HICAP and everywhere cells) firing interneurons because the failure rate was different across but not within these populations ***A left,*** is a schematic of a paired recording between a single interneuron and granule cell. ***A right***, shows a representative trace of an evoked action potential in the presynaptic interneuron and the subsequent eIPSC in a postsynaptic granule cell from a vehicle-treated culture. Bar graphs reveal no significant changes in eIPSC failure rate in granule cells following repeated action potential generation in either ***B,*** high-frequency firing interneurons (axo-axonic and basket cells) or ***C,*** adapting firing interneurons (HICAP, HIPP and everywhere cells) following chronic NMDAR inhibition. Note the difference in the y-axis scale in B and C. *Abbreviations:* GC, granule cell; IN, interneuron.

Figure 5. Paired-pulse ratio

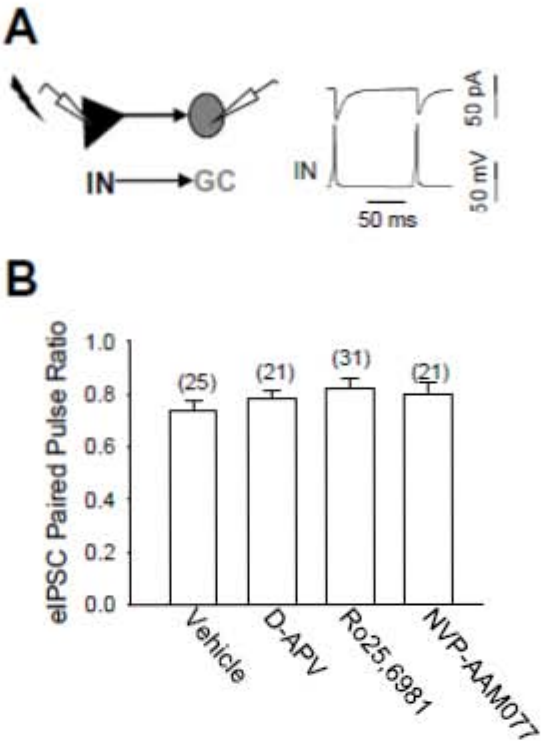


Figure 5. The paired-pulse ratio of evoked IPSCs (IPSCs) was similar in granule cells from cultures treated with vehicle and NMDAR antagonists. Presynaptic interneurons were current-clamped at their resting membrane potential and two brief 5 ms injections of depolarizing currents minimally sufficient to evoke a single action potential with the interval of 100 ms were applied to elicit two single action potentials while postsynaptic granule cells were voltage-clamped at -70 mV to record evoked IPSCs. The stimulation was repeated 50 times at a frequency of 0.3 Hz for each neuron pair. Data from all pairs were compiled because paired-pulse ratios were not significantly different between distinct populations of D/H border interneurons. ***A left,*** a schematic of a paired recording from an interneuron to a granule cell and ***A right,*** a representative trace of a pair of evoked action potentials in a presynaptic interneuron and subsequent a pair of evoked IPSCs in a postsynaptic granule cell from a vehicle-treated culture. ***B,*** bar graph reveals no significant difference in the paired-pulse ratio of evoked IPSCs between individual interneurons and granule cells in different treatment groups. Data were grouped for all D/H interneurons because there was no significant difference between distinct types of interneurons (data not shown). GC, granule cell; IN, interneuron. The number of slice cultures is indicated in parentheses.

Figure 6. Effect of $GABA_BR$ and mGluR on sIPSCs

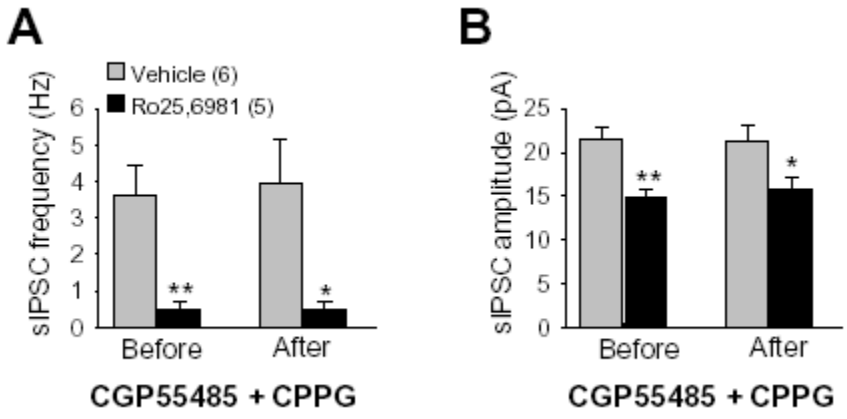


Figure 6. The frequency and amplitude of sIPSCs in granule cells were unchanged after blockade of $GABA_B$ receptors and group III metabotropic glutamate receptors (mGluR). Spontaneous IPSCs were recorded as described in the methods and Fig.1. Bar graphs revealed that acute blockade of $GABA_BR$ with CGP55845 (3 μM) and group III mGluR with CPPG (200 μM) did not affect sIPSC ***left,*** frequency or ***right,*** amplitude in granule cells from cultures treated with vehicle or Ro25,6981. Legend in ***A*** applies to ***A*** and ***B***. The number of granule cells / slice cultures is indicated in parentheses; *, $p<0.05$; **, $p<0.01$, than vehicle, t-test.

Figure 7. Effect of Kv channels on sIPSCs

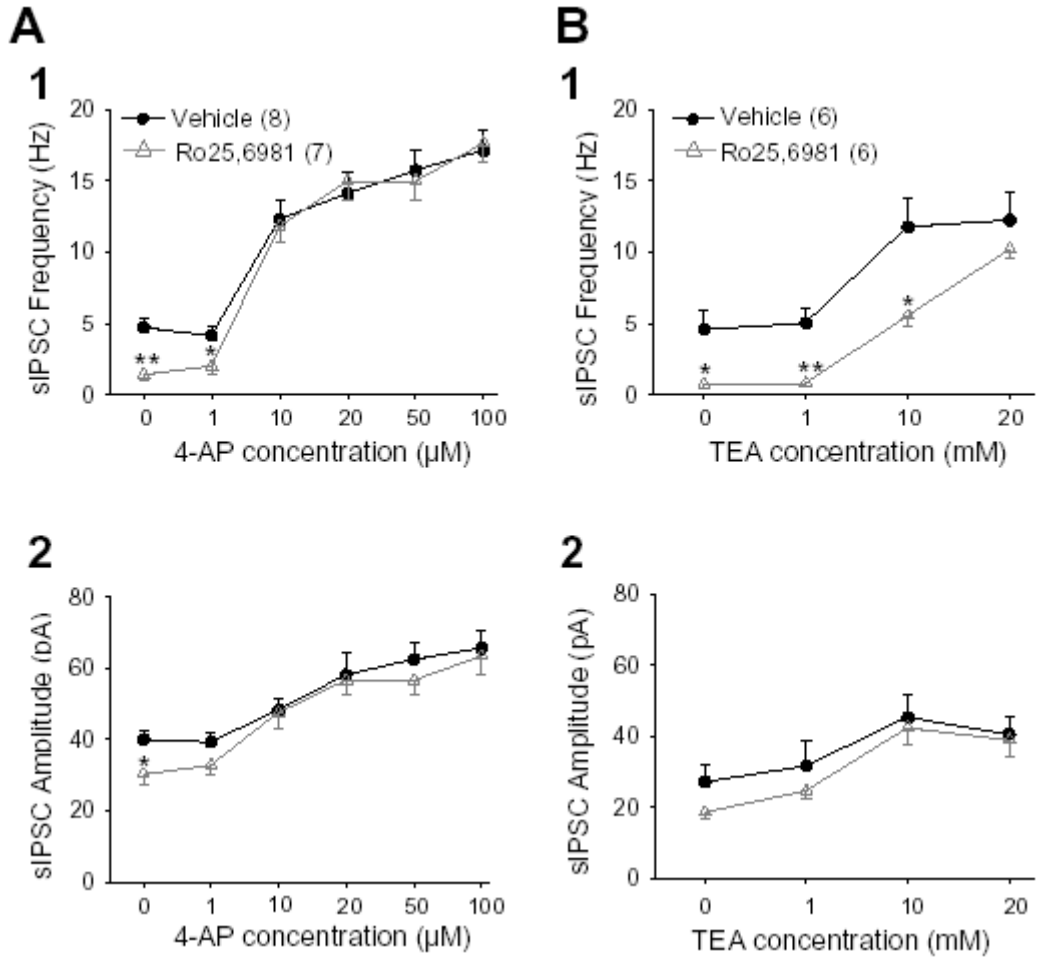


Figure 7. Two broad acting voltage-gated potassium channel antagonists abolished the difference in sIPSCs onto granule cells in vehicle- and Ro25,6981-treated cultures. sIPSCs were recorded as described in the methods. Concentration-response curves showed that as little as 10 μM 4-aminopyridine (4-AP) abolished the differences in sIPSC ***A1,*** frequency and ***A2,*** amplitude. Concentration-response curves showed that as little as 20 mM tetraethylammonium (TEA) abolished the differences in sIPSC ***B1,*** frequency and ***B2,*** amplitude between vehicle- and Ro25,6981-treated cultures. Legend in A1 applies to A1-2; in B1 applies to B1-2. The number of slice cultures is indicated in parentheses; *, $p<0.05$; **, $p<0.01$, different than vehicle, t-test.

Figure 8. Effect of Kv1 channels on sIPSCs

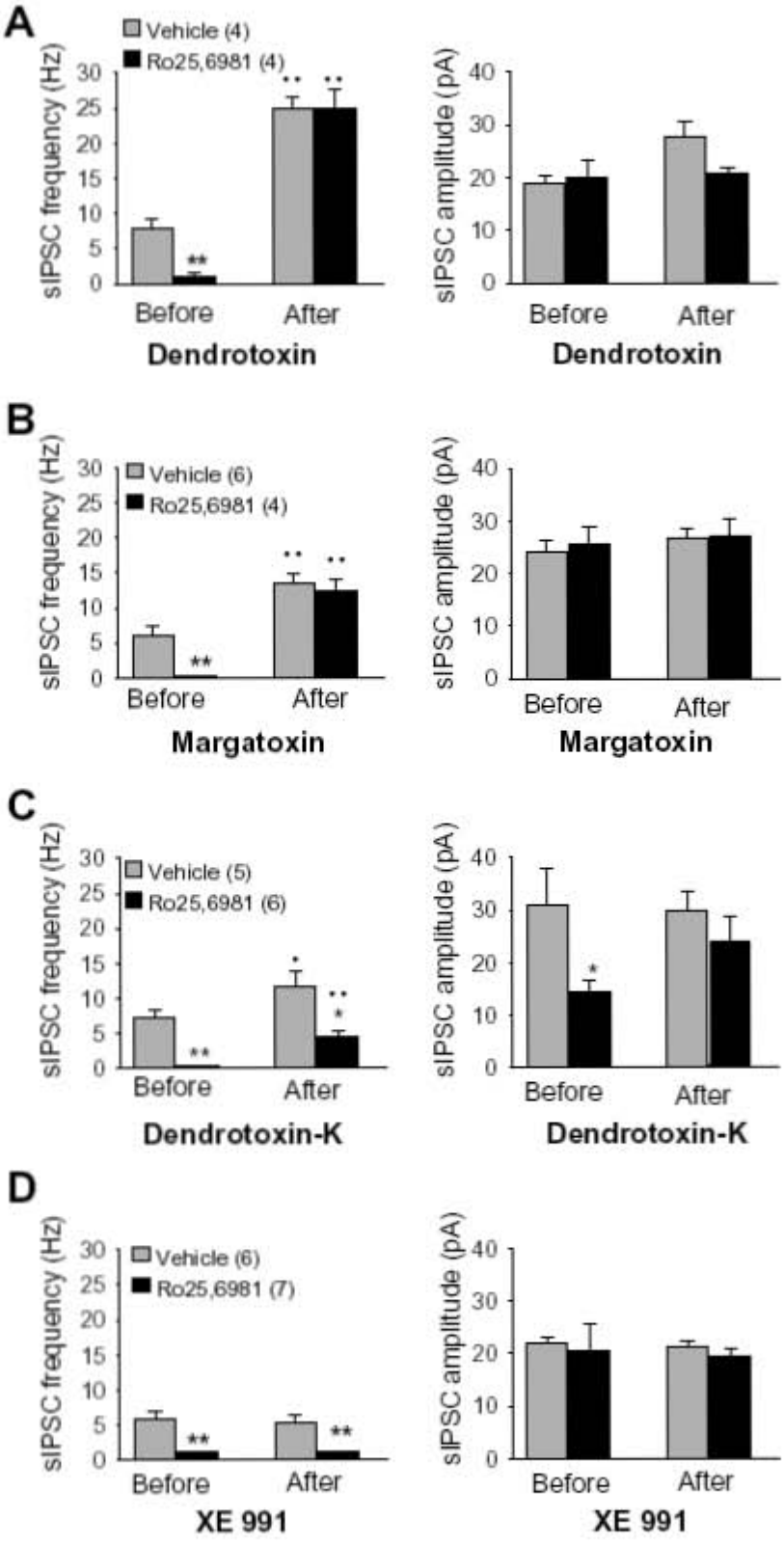
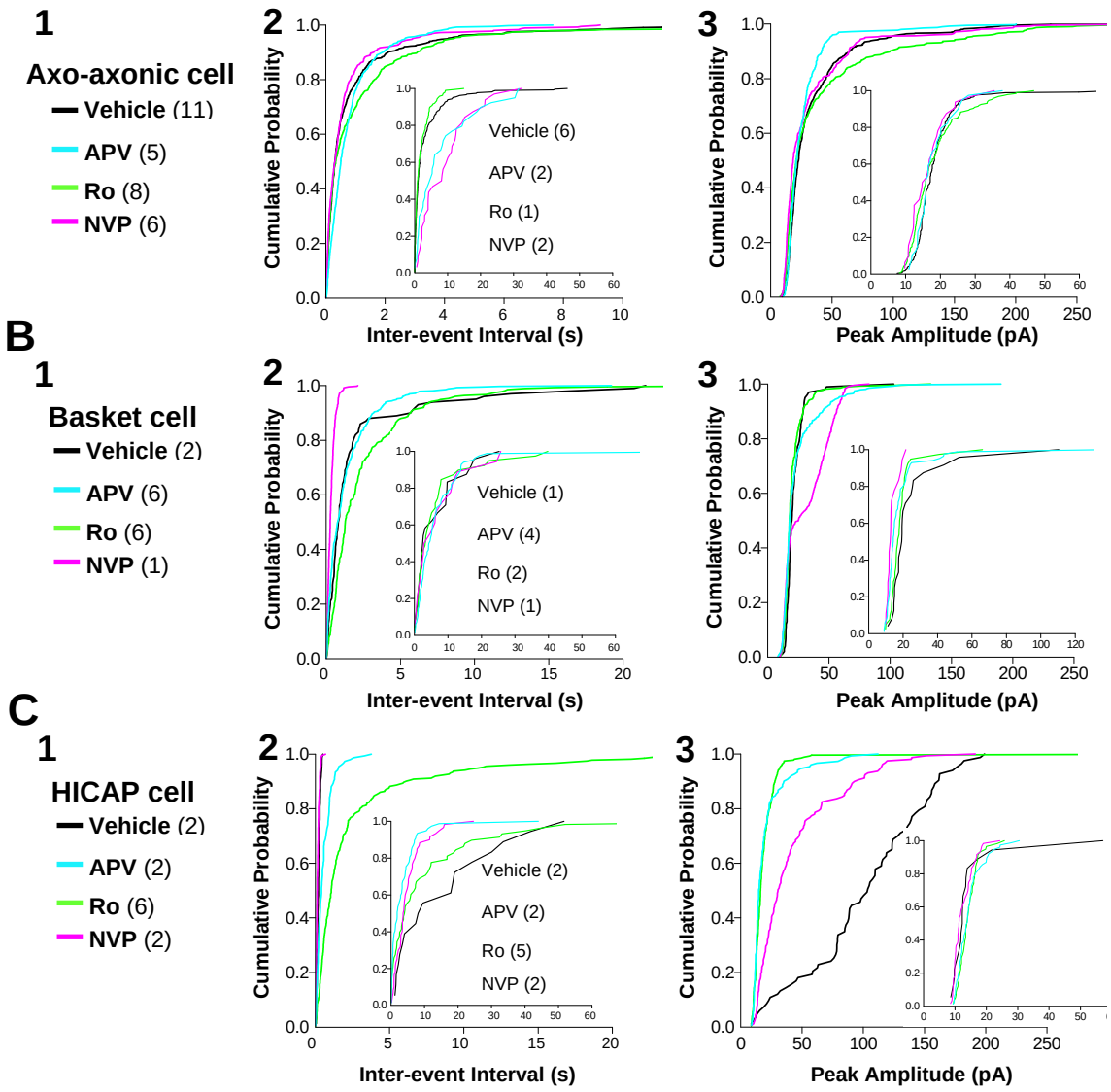
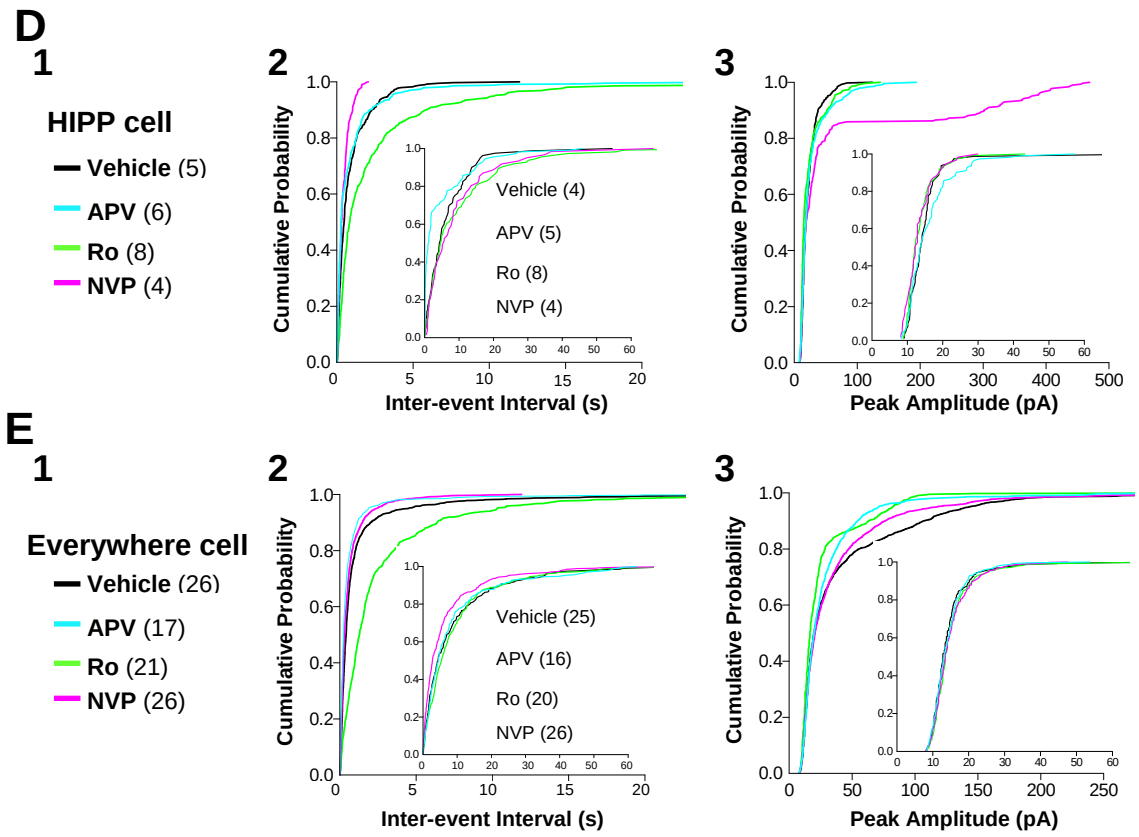


Figure 8. Acute blockade of Kv1, but not Kv7 channels occluded reductions in sIPSC frequency following chronic inhibition of NR2B-containing NMDAR. Recordings of sIPSCs in granule cells were conducted as described in the methods and Fig.1. Acute application of Kv1 channel blockers ***A,*** dendrotoxin (200 nM) or ***B,*** margatoxin (10 nM) occluded the difference between chronic vehicle and Ro25,6981 treatment in ***A,B left,*** sIPSC frequency, but had no significant effect on ***A,B right,*** sIPSC amplitude. Acute application of ***C,*** dendrotoxin-K (100 nM) increased ***C left,*** sIPSC frequency in granule cells from both vehicle- and Ro25,6981-treated cultures, but did not occlude the difference between chronic vehicle and Ro25,6981 treatment and had little effect on ***C right,*** sIPSC amplitude. Acute blockade of Kv7 channels with ***D,*** XE991 (10 µM) had no effect on ***D left,*** sIPSC frequency or ***D right,*** amplitude in granule cells from either vehicle- and Ro25,6981-treated cultures. Legend in ***A-D left*** applies to ***A-D left*** and ***right***. Before denotes measurements taken before acute application of blockers; After, denotes measurements taken before acute application of blockers in the same cells. The number of granule cells / slice cultures is indicated in parentheses; *, $p<0.05$; **, $p<0.01$, different than vehicle, t-test. Δ, $p<0.05$; ΔΔ<0.01, different than before in same chronic treatment group, paired t-test.

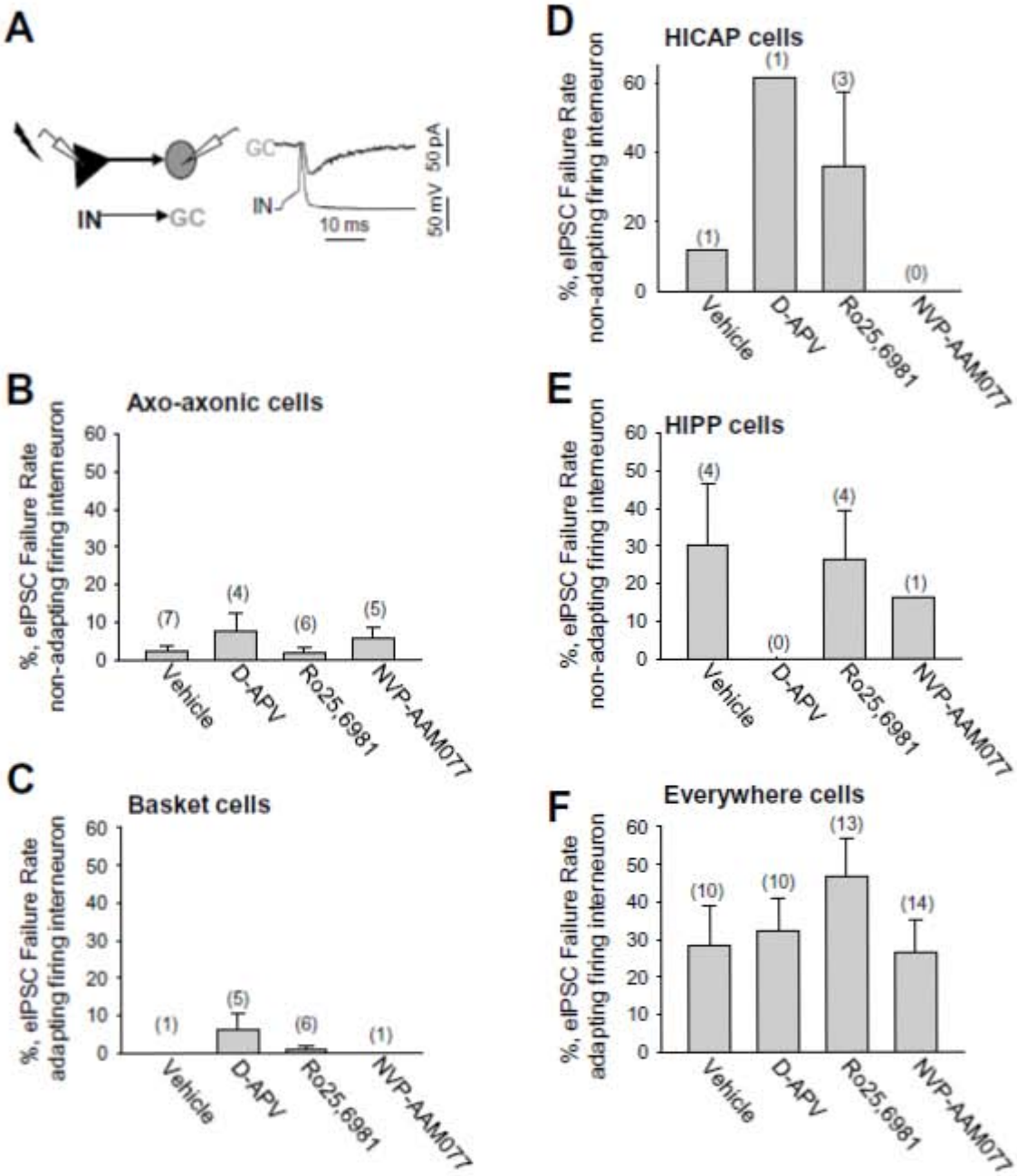
Supplemental Figure 1. IPSCs in interneuron subtypes





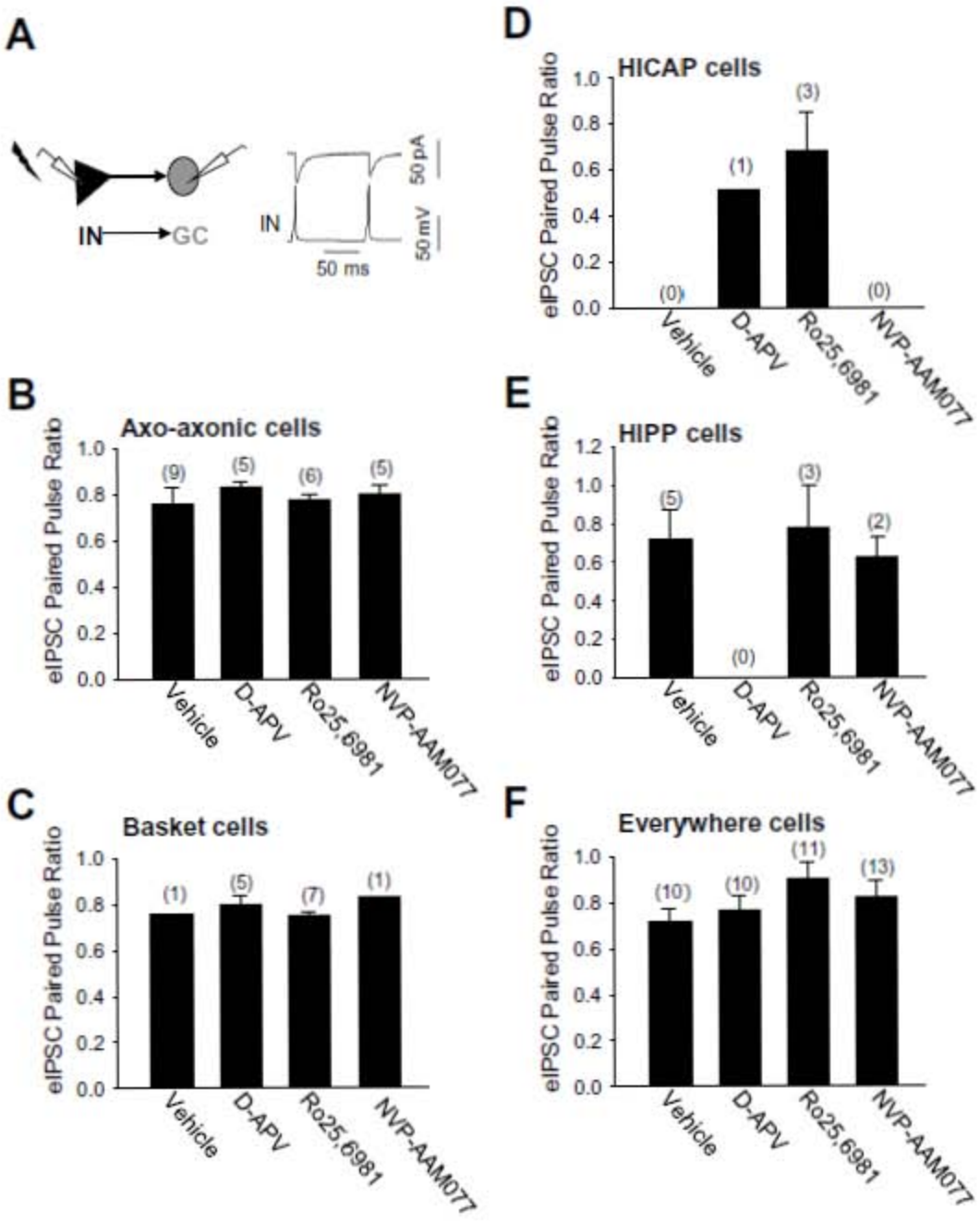
Supplemental Figure 1. Chronic inhibition of NR2B-containing NMDARs significantly reduced spontaneous, but had no effect on miniature IPSC frequency in D/H border interneuron subtypes. Spontaneous IPSCs were recorded at a -70 mV holding potential in recording buffer containing D-APV (50 μM) and CNQX (10 μM). Miniature IPSCs were recorded after subsequent addition of TTX (1 μM). Cumulative graphs revealed significant reductions in sIPSC frequency and modest alterations in sIPSC amplitude in ***A,*** axo-axonic cells, ***D,*** HIPP cells and ***E,*** everywhere cells except ***B,*** basket cells and ***C,*** HICAP cells following chronic inhibition of NR2B-containing NMDARs because too few replicates precluded meaningful statistic analyses. Legend in ***1*** applies to ***2-3*** in the same figure; the number of interneurons / slice cultures is indicated in parentheses for sIPSCs. **Inserts,** cumulative graphs show slight alterations in mIPSC frequency and amplitude in all D/H border interneuron types; the number of interneurons / slice cultures is indicated in parentheses for mIPSCs.

Supplemental Figure 2. Failure rate of eIPSCs in interneuron subtypes



Supplemental Figure 2. The failure rate of evoked IPSCs (eIPSCs) in granule cells following repeated action potential generation in D/H border interneuron subtypes was not significantly altered in cultures treated with NMDAR antagonists. *A left,* is a schematic of a paired recording between a single interneuron and granule cell. *A right*, shows a representative trace of an evoked action potential in the presynaptic interneuron and the subsequent eIPSC in a postsynaptic granule cell from a vehicle-treated culture. Bar graphs reveal no significant changes in eIPSC failure rate in granule cells following repeated action potential generation in *B,* axo-axonic cells and *F,* everywhere cells from cultures treated with NMDAR antagonists except *B,* basket cells, *C,* HICAP cells and *E,* HIPP cells because too few replicates precluded meaningful statistic analyses. The number of slice cultures is indicated in parentheses. *Abbreviations:* GC, granule cell; IN, interneuron.

Supplemental Figure 3. Paired-pulse ratio in interneuron subtypes



Supplemental Figure 3. The paired pulse ratio (PPR) of evoked IPSCs (IPSCs) following repeated action potential generation in D/H border interneuron subtypes was similar in granule cells from cultures treated with vehicle and NMDAR antagonists. ***A left,*** a schematic of a paired recording from an interneuron to a granule cell and ***A right,*** a representative trace of a pair of evoked action potentials in a presynaptic interneuron and subsequent a pair of evoked IPSCs in a postsynaptic granule cell from a vehicle-treated culture. Bar graphs reveal no significant changes in eIPSC PPR in granule cells following repeated action potential generation in ***B,*** axo-axonic cells and ***F,*** everywhere cells from cultures treated with NMDAR antagonists except ***B,*** basket cells, ***C,*** HICAP cells and ***E,*** HIPP cells because too few replicates precluded meaningful statistic analyses. The number of slice cultures is indicated in parentheses. *Abbreviations:* GC, granule cell; IN, interneuron.

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CHAPTER 4

Summary of results

Our lab previously documented the effects of chronic inhibition of NMDAR with different classes of NMDAR antagonists on seizure expression in hippocampal slice cultures. Chronic treatment with the high affinity competitive antagonist D-APV increased the total duration of seizures involving dentate granule cells induced by the $GABA_AR$ antagonist (BMI, 10 μM) or removal of Mg^{2+} block (0 mM Mg^{2+}). Conversely, chronic treatment with the NR2B antagonist significantly reduced the total duration of BMI- or 0 mM Mg^{2+}-induced seizures. Chronic inhibition of NMDAR with the uncompetitive moderate affinity antagonist showed a trend toward increased BMI-induced seizures (Wang and Bausch, 2004). The effect of chronic treatment with the NR2A antagonist on seizure expression remains under investigation. The goal of my thesis project was to investigate the factors that might contribute to neuronal hyperexcitability of dentate granule cells associated with changes in seizure expression following chronic inhibition of NMDARs with different classes of antagonists in hippocampal slice cultures. Principal findings for each NMDAR antagonists can be summarized as follows.

High affinity competitive NMDAR antagonist, D-APV

Following chronic blockade of NMDAR with the high affinity competitive antagonist D-APV, we found no significant changes in resting membrane potential or input resistance but slightly more negative action potential threshold in granule cells, similar to my previous data showing a trend toward a more negative action potential threshold (Bausch

et al., 2006). While this small shift in action potential threshold is unlikely to contribute to altered seizures by itself, it could act in concert with other pro-excitatory changes in D-APV-treated cultures. We also found: 1) increased mEPSC and $mEPSC_{AMPAR}$ frequency, amplitude and charge transfer; 2) a trend toward increased $sEPSC_{large}$ duration; 3) increased vGlut1-positive contacts onto granule cell dendrites; and 4) a trend toward increased mossy collaterals in the molecular layer. These findings suggested increased connectivity in excitatory networks. In contrast, 1) sIPSC and mIPSC amplitude and frequency in dentate granule cells and interneurons, 2) action potential and membrane properties of hilar interneurons and 3) GABA release probability were not dramatically altered as well following chronic NMDAR blockade with D-APV, suggestive of unchanged inhibitory inputs onto granule cells. Thus, increased connectivity in excitatory networks would shift the balance between excitation and inhibition toward excitation, and together with a more negative action potential threshold in granule cells (Chapter 2 and 3) could account for increased BMI- and 0mM Mg^{2+}-induced seizures following chronic NMDAR blockade with D-APV (Wang and Bausch, 2004; Dong and Bausch, 2005).

Moderate affinity uncompetitive NMDAR antagonist, memantine

Currently, memantine is a clinically well-tolerated therapeutic agent for dementia from moderate to severe Alzheimer's disease in the US (Mount and Downton, 2006) as well as Parkinson's disease in Europe (Lipton et al., 2007). Consistently, my study reports only slight alterations in excitatory circuits in the dentate gyrus following chronic treatment with the moderate affinity uncompetitive antagonist memantine. These changes include modestly increased mEPSC amplitude and charge transfer, a trend toward increased

mossy fiber collaterals in the molecular layer, and increased vGlut1-positive contacts onto granule cell dendrites. These rather limited pro-excitatory changes would slightly shift the balance between excitation and inhibition toward an increase in excitability, which may explain in part increased seizure susceptibility following chronic treatment with memantine (Loscher, 1998; Wang and Bausch, 2004). These findings are also consistent with previous studies reporting that memantine was ineffective in prevention of seizures in animal models (Meldrum et al., 1986; Loscher and Honack, 1990; Parsons et al., 1995).

NR2A-selective antagonist, NVP-AAM077

Following chronic inhibition of NR2A-containing NMDAR with NVP-AAM077, we only found significantly reduced $mEPSC_{NMDAR}$ charge transfer as well as a more negative action potential threshold in granule cells and increased action potential half-width, rise time and decay time in a subset of D/H border interneurons. Reduced $mEPSC_{NMDAR}$ charge transfer would decrease neuronal excitability, whereas a more negative action potential threshold would increase neuronal excitability. Thus, contribution of these changes to excitability in excitatory circuits remains unclear. Increased action potential half-width, rise time and decay time in interneurons could prolong synaptic terminal depolarization and thus increase the number of vesicles released by an action potential. On the other hand, these changes also could decrease firing rate and then result in a decrease in overall vesicle release. The dual effect on vesicle release may explain why sIPSC frequency was not significantly altered following chronic treatment with NVP-AAM077 in this study. No significant alterations in membrane properties, sEPSCs or

sIPSCs in granule cells suggested no dramatic changes in excitability of granule cells. Although a large body of articles showed the absence of selectivity and specificity of NVP-AAM077 for NR2A-containing NMDAR at a high concentration, the concentration of NVP-AAM077 (50 nM) that was selected in this study was previously shown to highly and preferentially inhibit NR2A-containing NMDARs (12-fold affinity for NR2A than NR2B) (Neyton and Paoletti, 2006). This concentration could block ~70% NR2A-containing NMDAR-mediated currents (Neyton and Paoletti, 2006). Therefore, it is unlikely that our observations resulted from chronic inhibition of non-NR2A-containing NMDARs and suggested that the effects of chronic treatment with NVP-AAM077 were due to partial inhibition of NR2A-containing NMDARs. Whether complete blockade of NR2A-containing NMDARs will exert different impacts on synaptic connectivity needs to be examined in the future.

NR2B-selective antagonist, Ro25,6981

Following chronic inhibition of NR2B-containing NMDAR with Ro25,6981, we found reduced $sEPSC_{large}$ frequency and decreased mossy fiber collaterals in the CA3 region as well as increased mEPSC frequency, amplitude and charge transfer. Since a recurrent granule cell–CA3 pyramidal cell excitatory circuit contributes to epileptiform events in organotypic hippocampal slice cultures (Bausch and McNamara, 2000), fewer mossy fiber collaterals in the CA3 region might account for reduced $sEPSC_{large}$ frequency due to reduced granule cell inputs to CA3. Paired recording revealed that $sEPSCs_{large}$ mirrored epileptiform bursts recorded simultaneously with field potential recordings. Thus, reduced $sEPSC_{large}$ frequency is consistent with decreased excitability in excitatory

circuits and reduced BMI-induced seizure number (Wang and Bausch, 2004). Increased mEPSC frequency suggested increased release probability and/or the number of synapses, whereas increased mEPSC amplitude and charge transfer implicated increased clusters of postsynaptic receptors. Our data suggest that changes in individual synapses onto granule cells could not account for reduced seizure expression following chronic Ro25,6981 treatment.

In addition, we documented opposing effects of chronic inhibition of NR2B-containing NMDAR on synaptic and extrasynaptic NMDAR function. Reduced NMDAR-mediated mEPSC frequency and charge transfer suggested diminished postsynaptic NMDAR function, whereas increased NMDAR-mediated tonic current amplitude implicated enhanced extrasynaptic NMDAR function in granule cells following chronic Ro25,6981 treatment. The opposite effects of chronic Ro25,6981 treatment on synaptic and extrasynaptic NMDAR-mediated currents suggest movement of synaptic NMDAR into extrasynaptic membranes. Reduced postsynaptic NMDAR function might contribute to reduced seizures following chronic treatment with Ro25,6981. However, whether increased extrasynaptic NMDAR function contributed to neuronal excitability remains elusive because the functional consequence of tonic NMDA currents is currently unclear (for more detailed discussion, see Chapter 2).

Finally, we found dramatically reduced sIPSC frequency and a trend toward decreased sIPSC amplitude following chronic inhibition of NR2B-containing NMDAR (see schematic Fig. 1). Since GABAergic inhibition can balance excitation and synchronize neuronal firing, contribution of changes in sIPSCs to seizures needs to be considered from both aspects. In mature dentate granule cells, GABAergic transmission

may play a more prominent role in neuronal synchronization than in balancing excitation (Vida et al., 2006). Thus, diminished action potential-dependent GABAergic transmission might reduce synchronization of neuronal firing and then result in reduced seizure expression involving dentate granule cells following chronic inhibition of NR2B-containing NMDARs. The discussion is described in more detail in Chapter 3.

Overall Discussion and Summary

My study shows opposite effects of chronic treatment with D-APV and with Ro25,6981 on glutamatergic network activity onto dentate granule cells (large amplitude sEPSCs). Moreover, only chronic treatment with Ro25,6981 increased extrasynaptic NMDAR-mediated tonic currents in granule cells, and reduced GABAergic network activity onto granule cells and interneurons. These findings are unlikely to be explained by the degree of NMDAR inhibition because chronic inhibition of NR2A-containing NMDAR with NVP-AAM077 and with memantine did not mimick the effect of chronic inhibition of NR2B-containing NMDAR. Nevertheless, these findings are somewhat consistent with a previous study showing opposite effects of short-term treatment with ifenprodil (30 mins, an analog of Ro25,6981) and with DL-APV on surface GluR1 expression in cultured hippocampal neurons (Kim et al., 2005). Meanwhile, Kim et al. have proposed that the specific coupling of NR2B to SynGAP, which inhibits the ERK pathway, might underlie the effect of treatment with ifenprodil on surface GluR1 expression. Although this explanation is appealing, it raises a question: why did treatment with DL-APV have opposite effect on GluR1 surface expression as DL-APV also blocks NR2B-containing NMDAR? Thus, simple inhibition of NR2B-containing NMDARs might not be sufficient

to explain specific effects of chronic treatment with Ro25,6981 or ifenprodil on network activity and tonic NMDAR-mediated currents.

Unlike competitive NMDAR antagonists, Ro25,6981 is an allosteric noncompetitive NR2B antagonist that has a different binding site from an agonist. Hence, Ro25,6981 can bind to NMDA receptors with an agonist simultaneously. The allosteric binding can produce conformational changes in receptors and sometimes leads to an alteration in potency for agonists (Burgen, 1981; Kenakin, 2004b). For instance, a previous study showed that Ro25,6981 (1 μM) could potentiate the responsiveness of NMDAR receptors to 1 μM NMDA, but inhibited ≥90% NMDAR-mediated currents activated by 100 μM NMDA (Fischer et al., 1997). This suggests that Ro25,6981 may act as a modulator of NR2B-containing NMDA receptors. This allosteric modulator Ro25,6981 may potentiate NMDAR activity while hypoactivated, whereas inhibit NMDAR while hyperactivated, thereby maintaining the physiological level of NMDAR activity. By contrast, the competitive NMDAR antagonists, D-APV and NVP-AAM077, inhibit the responsiveness of NMDA receptors to low concentration of agonists to a greater degree than to high concentration of agonists. The moderate affinity uncompetitive NMDAR antagonist, memantine, blocks open NMDAR channels, but its inhibitory efficacy independent of agonist concentration. Unlike the allosteric antagonist Ro25,6981, the competitive antagonists and channel pore blocker just block or inhibit rather than modulate NMDAR activity. Hence, the allosteric pharmacological property of Ro25,6981 may explain the specific effects of chronic treatment with the NR2B subunit-selective antagonist but not with other classes of NMDAR antagonists on neuronal network activity.

Currently, allosteric antagonists or modulators are of special interest as potential therapeutic agents for treating diseases because of their unique properties (Kenakin, 2004a). Results from this study might provide a rational basis for the development of allosteric noncompetitive NR2B antagonists as therapeutic treatments for epilepsy. In addition, my study showed that functional upregulation of Kv1 channels was involved in the effect of chronic treatment with Ro25,6981 on GABAergic network activity. If diminished GABAergic network activity contributed to reduced seizure expression following chronic Ro25,6981 treatment, allosteric modulators that potentiate Kv1 channel function may be also potential agents for treating epileptogenesis.

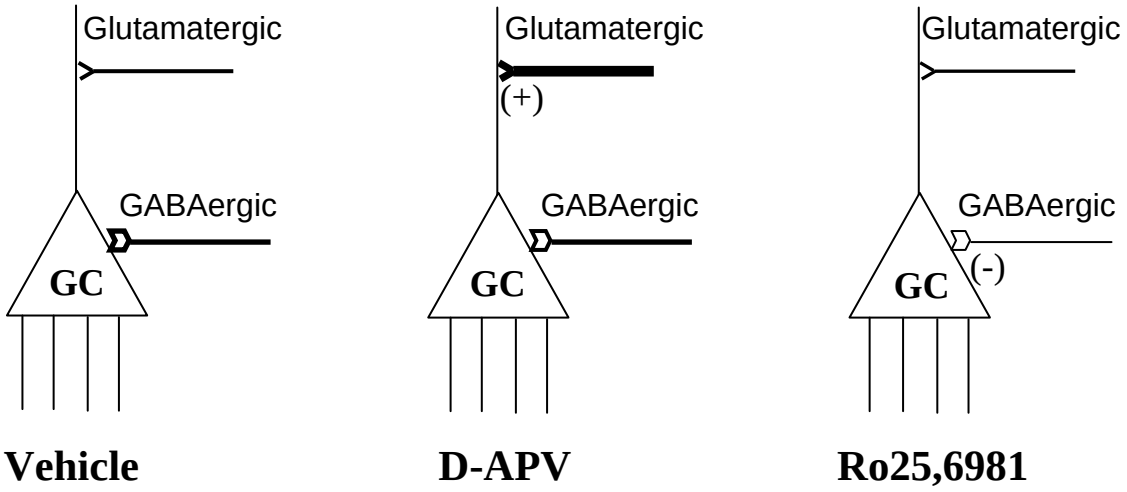

Figure 1. Schematic of glutamatergic and GABAergic transmission onto individual dentate granule cells from vehicle-, D-APV-, and Ro25,6981-treated cultures. Overall, chronic inhibition of NMDAR with D-APV significantly increased glutamatergic transmission with minimal changes in GABAergic transmission onto individual granule cells. On the contrary, chronic inhibition of NR2B-containing NMDAR with Ro25,6981 profoundly reduced GABAergic transmission, but had no dramatic effect on glutamatergic synaptic transmission. GC, granule cell.

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