Published in final edited form as:

Mol Cell Neurosci. 2009 August ; 41(4): 383–396. doi:10.1016/j.mcn.2009.04.003.

Knockdown and overexpression of NR1 modulates NMDA receptor function

Maggie L. Kalev-Zylinska¹, Wymond Symes^{1,2}, Deborah Young^{1,3}, and Matthew J. During^{1,4,*}

¹ Department of Molecular Medicine and Pathology, The University of Auckland, Auckland, New Zealand ² Catalyst™ Research and Development Ltd, Katikati, New Zealand ³ Department of Pharmacology and Clinical Pharmacology, The University of Auckland, Auckland, New Zealand ⁴ Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, OH, USA

Abstract

The *N*-methyl-D-aspartate receptor (NMDAR) is critically involved in learning and memory, neuronal survival, as well as neuroexcitotoxicity and seizures. We hypothesize that even mild reductions in the numbers of hippocampal NMDARs could impair learning and memory, whereas increasing receptor activity would facilitate learning but reduce seizure threshold. We developed novel gene transfer strategies assisted by an adeno-associated viral vector 1/2 to bi-directionally modulate expression levels of the NR1 protein in rat hippocampus. Functional consequences of the altered NR1 expression were examined in the acute seizure model, and on normal processes of fear memory and neurogenesis. We found that knocking down NR1 protected against seizures at the expense of impaired learning, as predicted. Paradoxically, NR1 overexpression not only increased fear memory and neurogenesis, but also delayed onset of more severe seizures. In conclusion, the observed consequences of NR1 knockdown and overexpression underscore NMDAR requirement for neuronal plasticity, and are in agreement with its dichotomous functions.

Introduction

States associated with abnormalities in the *N*-methyl-D-aspartate receptor (NMDAR) function are central to the pathogenesis of common human neurological disorders. The NMDAR mediated excitotoxicity represents a major mechanism of neurodegeneration in many disease states (for review, see Aarts and Tymianski, 2004). In human epilepsy, rising levels of extracellular glutamate precede the onset of seizures (During and Spencer, 1993). Consistently in animal epilepsy models, strategies that reduce NMDAR expression inhibit seizures induced by acoustic, electrical or chemical stimuli (Chapman et al., 1996; Haberman et al., 2002; Zapata et al., 1997). The administration of kainate represents a common experimental protocol to model seizures in animals (Ben-Ari, 1985). One of the consequences of kainate action is the enhancement of glutamate release from the pre-synaptic terminal, which in turn overactivates post-synaptic NMDARs (Chittajallu et al.,

*Corresponding author: Matthew J. During, Molecular Virology, Immunology & Medical Genetics, The Ohio State University, Columbus, OH 43210, USA, during.1@osu.edu, phone: 614. 247. 4351, fax: 614. 688. 0731.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1996). Pharmacological inhibition of NMDARs in this context prevents both seizures and cell death, indicating that functional NMDARs participate in causing neuronal injury (Brandt et al., 2003; Clifford et al., 1990),.

Although NMDAR antagonists inhibit seizures in humans, they unexpectedly failed as neuroprotective agents in patients with head injury and stroke (Lees et al., 2000; Morris et al., 1999; White et al., 2007). It appears that the critical involvement of NMDARs in normal neuronal processes, including their increasingly recognized contribution towards neuroprotection, presents a challenge for safely manipulating NMDAR function in the diseased brain. Growing experimental evidence indicates that the physiological activity of synaptic NMDARs promotes neuronal survival (Ciani et al., 1997; Ikonomidou et al., 1999; Ikonomidou et al., 2000). The NMDARs are also required for preconditioning responses that increase brain resistance to injury (Dirnagl et al., 2003; Kato et al., 1992). Furthermore, loss of NMDAR function in the hippocampus significantly impairs learning and memory (Huerta et al., 2000; McHugh et al., 2007; Nakazawa et al., 2002; Rampon et al., 2000; Tsien et al., 1996). Even if NMDAR expression becomes reduced in only a small number of hippocampal neurons, learning deficits ensue (Cheli et al., 2006). This suggests that there is not a high level of redundancy that applies to NMDAR function in the hippocampus, and therefore a window to safely knocking down the obligatory receptor subunit 1 (NR1) may be narrow. However, data on how NMDAR-dependent processes respond to the availability of NR1 are limited.

Somatic cell gene transfer can help define roles of NMDARs in different brain regions, ascertain contributions from its subunits, and characterize functional consequences of receptor manipulation. Hence, we have developed a novel gene transfer method to modulate NMDAR function *in vivo* (Kalev-Zylinska and During, 2007). Our strategies involved genetic overexpression and RNA interference-mediated knockdown of the NR1 subunit, with coding sequences delivered to rat hippocampus by the adeno-associated chimeric viral (AAV) vector 1/2. Our knockdown method reduced NR1 expression approximately by half, and NR1 overexpression increased NR1 protein levels 2–3-fold. Here, we report how these opposing manipulations of NR1 expression affected not only the pathology of acute kainate-induced seizures, but also physiological functions influenced by the hippocampal formation, in particular fear memory and neurogenesis.

Results

Characterization of the NR1 knockdown *in vitro*

We designed four short interfering RNA sequences (siRNAs) targeting rat *NR1* transcripts (siNR1) (Table 1). When transiently co-transfected into HEK293 cells together with its cDNA target, two of these sequences, designated here as siNR1-2 and siNR1-3, reliably down-regulated NR1 expression (Fig. 1A–C). The highest level of knockdown was seen with the siNR1-3 molecule ($66 \pm 5\%$; $p < 0.005$) (Fig. 1C), complementary to nucleotides 1561 – 1582 in the rat *NR1* cDNA (X63255).

To enable their use *in vivo*, short-hairpin (sh) precursor sequences encoding siNR1-3 were cloned into a bicistronic expression cassette, pAM–shNR1-3–dYFP, in which the dYFP (destabilized yellow fluorescent protein) served as a marker of expression. We confirmed that this precursor construct remained effective in knocking down NR1 (Fig. 1D and E). HEK293 cells stably expressing the NR1 protein were generated and transiently transfected with the pAM–shNR1-3–dYFP. Three days later, FACS was used to sort cells according to the dYFP fluorescence (Fig. 1D). Examination of their protein lysates revealed that in cells expressing the knockdown cassette (i.e. dYFP positive), NR1 protein levels declined by $50 \pm 5\%$ ($p < 0.05$), compared to the effects of a control plasmid expressing enhanced green

fluorescent protein (eGFP) alone (Fig. 1E). We also confirmed that the siNR1-3 sequences did not interfere with the expression of two other NMDAR subunits, NR2A and NR2B, when tested in transiently co-transfected HEK293 cells by immunocytochemistry (Fig. 1F and G) and semi-quantitative immunoblots (Fig. 1H and I).

Modulation of NR1 expression in vivo

A method of gene transfer assisted by the recombinant AAV 1/2 vector was used to deliver the NR1 knockdown or overexpression sequences to rat hippocampi. The design of packaged NR1 and shNR1 expressing cassettes was as previously reported (Kalev-Zylinska and During, 2007). Our previous work validated the shNR1 sequences against a knockdown control targeting dYFP (i.e. shdYFP). In the current study, consequences of the modulated NR1 expression were compared against a control vector expressing eGFP.

When delivered into the dorsal hippocampus, all vectors efficiently transduced the target region as assessed at 6 weeks by NR1 immunohistochemistry (Fig. 2A–C). The semi-quantitative analysis of immunoblots estimated the increase in NR1 expression at 2.5–3-fold higher, compared to eGFP controls (Fig. 2D). This level of NR1 expression was associated with a $1.5[\pm 0.1]$ -fold increase in NR2B levels [$p < 0.05$], but no obvious change in the expression of NR2A (Fig. 2D). In comparison, the RNA interference strategy reduced NR1 protein levels in the dorsal hippocampus by $53 \pm 7\%$ (Kalev-Zylinska and During, 2007).

To precisely determine how our strategies affected *NR1* expression at the transcript level, granule cell layers were excised from the hippocampal dentate gyrus by laser microdissection (Fig. 2E). The dissected tissue was examined by real-time RT-PCR, and we found that *NR1* transcript levels in the shNR1-transduced brains were reduced by $55 \pm 10\%$ ($p < 0.05$) (Fig. 2F). In comparison, NR1 overexpression increased *NR1* mRNA to 300–400% ($p < 0.001$; Fig. 2F).

Effects of the modulated NR1 expression on kainate-induced seizures

Here, we tested consequences of the modulated NR1 expression on seizures in an intrahippocampal kainate model. Recombinant AAV 1/2 vectors expressing NR1, shNR1 or eGFP were delivered stereotactically to the dorsal hippocampus bilaterally ($n = 6$ rats per group) (Fig. 3A). Six weeks later, cannulae and electrodes were implanted in order to inject kainate directly into regions with previously modulated NR1 expression, or to monitor electroencephalography (EEG), respectively (Fig. 3B). We found that rats with increased NR1 expression did not differ in the overall number, latency or duration of EEG discharges from eGFP-injected controls (Fig. 3C). On the other hand, rats with reduced NR1 expression developed significantly fewer EEG seizures, and with longer latencies ($p < 0.05$). Representative examples of EEG traces for all groups are shown in Fig. 3D. The majority of seizures in eGFP- and shNR1- injected rats occurred in the first 60 min after kainate injection. No seizures were recorded in NR1 knockdown animals beyond 60 min of observation. In contrast, most EEG seizures in NR1-injected rats occurred during the second hour (Fig. 3D and Fig. 4).

Behavioral seizure phenotypes were characterized according to the Racine scale (Racine, 1972) (Fig. 4). Seizure stages II – V (head nodding, forelimb clonus, rearing and falling) occurred at similar frequencies in the eGFP- and NR1- injected rats (Fig. 4A). Nevertheless, rats with NR1 overexpression displayed longer latencies to stages II and III, compared with eGFP controls ($p < 0.05$). In contrast, there was no limb clonus nor generalized tonic-clonic convulsions in shNR1 rats, where all EEG seizures manifested solely as stage I (Fig. 4A and B). These included animal immobility, staring and facial movements. In the second hour of

observation, seizures resolved in shNR1-injected rats but persisted with NR1 or eGFP overexpression (Fig. 4C).

The effects on seizures observed in the acute kainate model implied that the NR1 knockdown technique effectively attenuated NMDAR function *in vivo*. In addition, a modest anti-epileptic effect on more severe seizures was observed with NR1 overexpression.

Kainate-induced neuronal damage

Rats were killed five days after kainate administration, and the extent of intrahippocampal damage was assessed by staining brain tissue with Fluoro-Jade B, a substance that visualizes injured or activated neurons (Schmued and Hopkins, 2000).

Consistent with known patterns of kainate toxicity, we found that all eGFP-injected hippocampi contained Fluoro-Jade B - positive pyramidal cells either in the Cornu Ammonis (CA) 3 field or CA3 and CA1 (Fig. 5A). In the NR1-injected brains, a variable amount of neuronal damage was seen. This ranged from the presence of Fluoro-Jade B - positive neurons at the site of kainate injection only, to signs of generalized damage involving both CA3 and CA1. Overall, the extent of Fluoro-Jade B staining in NR1-injected animals paralleled their severity of seizures. In rats with hippocampal NR1 knockdown, tissue damage was predominantly confined to the sites of kainate injection (Fig. 5A). We arbitrarily scored the extent of neuronal damage revealed by Fluoro-Jade B (Fig. 5B). Scores were lowest for rats injected with the knockdown construct, and a wider range of scores was obtained for NR1-injected animals.

To further examine toxicity induced by kainate, we looked for morphological abnormalities such as loss of pyramidal cell nuclei, pyknosis or nuclear vacuolization in the hippocampal sections stained with H&E (Fig. 5C). In all of the eGFP-injected brains, there was an obvious loss of pyramidal neurons in CA3 and most of CA1. Similarly, nuclei were lost in 4/6 CA3 regions in NR1-injected brains. However within CA1, nuclear pyknosis predominated over the loss of neurons in 5/6 NR1-injected hippocampi. In shNR1-injected brains, there were no obvious loss of nuclei and no significant pyknosis in CA3 and CA1. However, large nuclear vacuoles were common in CA3 pyramidal neurons (Fig. 5C).

Overall, kainate produced least amount of tissue damage in shNR1-injected hippocampi. Furthermore, some mild qualitative differences in the pattern of damage were observed between eGFP- and NR1- overexpressing brains.

Sites with reduced and enhanced NR1 expression before and after kainate

Here, we examined which cell types expressed the NR1 knockdown and overexpression cassettes after the unilateral delivery of AAV vectors into the dorsal hippocampus, first with no kainate administration and later after kainate-induced seizures.

Because the knockdown construct contained the dYFP marker, anti-dYFP immunohistochemistry was used as surrogate to identify sites with reduced NR1 expression. In comparison, neurons with NR1 overexpression were identified based on a strong NR1 immunohistochemistry signal (Fig. 6A and B). We saw that both constructs expressed well in the excitatory neurons – both pyramidal and granule. In contrast, spread to the interneurons was evident only with NR1 overexpression (Fig. 2C; Fig. 6A and B; Kalev-Zylinska and During, 2007). It is likely that the pattern of NR1 expression assisted by AAV 1/2 in this study influenced the effects of kainate we observed.

Others previously noted that reactive increases in NR1 expression arise in experimental animal and human epileptic tissue (Mathern et al., 1998; Rafiki et al., 1998). Hence, we examined if similar changes were present in rat brains following kainate-induced seizures.

We saw an obvious reduction in NR1 expression in areas coincident with neuronal loss, most evident in eGFP-injected brains (Fig. 6C). However, cells with increased NR1 expression were also found in eGFP-injected hippocampi, including in the regions of dentate gyrus, CA1 and CA3 (Fig. 6D). Similarly, in the context of NR1 knockdown, some granule and pyramidal cells, as well as occasional hilar interneurons, displayed enhanced NR1 expression (Fig. 6E). In the NR1-transduced tissue, kainate led to a reduction in NR1 expression in both granule and pyramidal cell layers. However, NR1 expression remained strong in interneurons, particularly within the hilar regions of dentate gyrus (Fig. 6F). These qualitative changes in NR1 expression after kainate suggest that modulation of NMDAR expression may play a role in cellular adaptation to seizures.

NR1 expression and fear memory

Independent experiments were conducted to explore how varying hippocampal NR1 expression would impact on two physiological functions of the hippocampus, fear memory and neurogenesis. Fear memory represents a form of a strong associative learning, where the neural circuitry is relatively well understood and it includes hippocampal formation and NMDARs (Maren, 1999; Melik et al., 2006).

In this experiment, AAV 1/2 vectors expressing NR1, shNR1 or eGFP sequences were delivered into the hippocampus unilaterally (on the right side of the brain, $n = 12$ rats per group). Twelve weeks later, effects on fear memory were examined using a simple and robust behavioral task of Passive Avoidance (Fig. 7A). During training rats develop an aversive association between the experimental apparatus and a 1 milliamp electric shock delivered to their feet when they enter the dark chamber. Before the foot-shock was given (on day 0), the time to enter the dark chamber did not differ between animals ($p = 0.1$) (Fig. 7B). We examined retention of the aversive association 24 h later, and then on days 3 and 7, until all rats entered the dark compartment. Most of the shNR1-injected rats (10 of 12) entered the dark on day 1 (Fig. 7C). In contrast, only 3 of the 12 NR1-overexpressing animals entered on that day ($p < 0.05$). The time delay to enter the dark chamber i.e. step-through latencies, increased following training for all groups, indicating that all rats developed memory for the aversive event ($p < 0.001$) (Fig. 7D). The probability of entry to the dark differed significantly between groups (Kaplan-Meier method; log rank test $p = 0.01$) (Fig. 7D). The median step-through latencies were longer for rats with NR1 overexpression, and shorter for animals with NR1 knockdown ($p < 0.05$; Kruskal-Wallis test, censored for each group) (Fig. 7D).

NR1 expression and neurogenesis

Neurogenesis takes place in the hippocampal dentate gyrus, contributing to plasticity of the adult brain, in a process known to be modulated by NMDARs (Tashiro et al., 2006).

Seven days after completion of Passive Avoidance testing, we administered BrdU to animals intraperitoneally (i.p.) ($n = 10$ per group) in divided doses over 3 days, to label cells that proliferated during that time. Half of the animals injected ($n = 5$ per group) were killed 24 h later to count the numbers of cells generated in the subgranular zone of the dentate gyrus. The remaining rats ($n = 5$ per group) were killed after a further 4 weeks to determine the proportion of BrdU-labeled cells that survived and acquired antigenic determinants of neuronal differentiation (Fig. 8).

We observed that in the subgranular zones with attenuated NR1 expression, the numbers of proliferating cells were reduced ($p < 0.05$) (Fig. 8A and B). On the other hand, NR1 overexpression did not change the rate of progenitor proliferation but increased numbers of BrdU-labelled cells that survived after 4 weeks ($p < 0.05$) (Fig. 8C and D). However, when these surviving cells were examined for the co-expression of the neuronal marker, NeuN, no difference was found between groups ($p = 0.1$) (Fig. 8E and F), suggesting that the level of NR1 expression did not have an effect on the degree of neuronal differentiation.

Discussion

In this work, we used an AAV 1/2 vector- mediated strategy to either overexpress or knock down the NR1 subunit of NMDAR in rat hippocampus. Consequences of these manipulations were studied in the acute kainate model, and on normal processes of fear memory and neurogenesis. Attempts to modulate NMDAR function *in vivo* are of interest as they may offer insights into the design of novel therapeutic interventions.

Hippocampal redundancy restricts applications of NR1 knockdown

Our strategy to knockdown NR1 in rat hippocampus markedly reduced seizure latency, number and severity. This is consistent with the effects others achieved with delivery of NR1 antisense (Chapman et al., 1996; Haberman et al., 2002; Zapata et al., 1997). In addition, we saw an associated decrease in the extent of kainate-induced neuronal damage. Lesser seizures likely limited the hippocampal injury, however damage declined not only in CA1 but also in CA3, raising a possibility that knocking down NR1 provided some protection to neurons in CA3. In support, NMDAR inhibitors restrict cell death in the kainate model, even if seizures continue (Brandt et al., 2003; Clifford et al., 1990). Others have shown that intrahippocampal administration of kainate induces direct toxicity in CA3 (Bouillere et al., 1999), and the model has been used to study potential neuroprotective interventions (Kaufer et al., 2004; Wang et al., 2005).

Of interest, our RNA interference approach led to only an approximate halving of hippocampal NR1 expression, yet when applied unilaterally resulted in significant impairment of hippocampal function. Specifically, shNR1-injected rats performed poorly in the Passive Avoidance task, and there was a reduction in the proliferation of neuronal progenitors in the subgranular zone of dentate gyrus. NMDAR-dependent processes are known to be critical for learning. Arresting NR1 expression in CA1 neurons impairs memory for spatial, temporal, olfactory, visual and emotional stimuli (Huerta et al., 2000; Rampon et al., 2000; Tsien et al., 1996). Similarly, deleting NR1 selectively from CA3 or dentate gyrus leads to memory defects (McHugh et al., 2007; Nakazawa et al., 2002). Furthermore, recent studies suggest that normal levels of the NR1 protein appear to be required in practically all hippocampal neurons (> 95%) to ensure normal learning (Cheli et al., 2006). Our experiments were designed to further explore this surprising phenomenon of low hippocampal redundancy. We manipulated NR1 expression on one (right) side of the hippocampus only, and tested fear memory in the Passive Avoidance paradigm. Our NR1 knockdown construct expressed well throughout the dorsal hippocampus, with some very limited spread ventrally and contralaterally (Kalev-Zylinska and During, 2007). Hence, we cannot comment on the contribution of the different hippocampal areas towards emotional memory processes, however a low redundancy phenomenon has been confirmed.

Taken together, although lowering NR1 expression may offer benefits in seizure models, side effects do occur even with the partial NR1 knockdown. Our data suggest that if NMDAR expression were to be manipulated by RNA interference for seizure control, such an approach would require tighter temporal regulation to reduce cognitive side effects.

Could NR1 overexpression be of advantage?

We did not expect to see the phenotypic effects that followed NR1 overexpression. This strategy produced no change in the number and duration of seizures, and no increase in cell death, suggesting a lack of pathological overactivation of NMDARs. In fact, some attenuation in seizures has occurred, manifested as longer latencies to Racine stages II and III.

The mechanism behind the subtle anti-epileptic phenotype seen with NR1 overexpression is unclear. However, studies show that in primary inhibitory interneurons, NMDAR activity drives dendro-dendritic inhibition (Christie et al., 2001). An inadvertent reduction of NR1 expression in these cells creates a state of hyperexcitability (Haberman et al., 2002). Our AAV 1/2-mediated transduction increased NR1 expression in pyramidal neurons, granule cells and interneurons. Following kainate administration this enhanced NR1 expression weakened in the excitatory neurons, but not in the interneurons. We will attempt to target and more closely characterize specific populations of interneurons transduced with NR1 in future studies. Here, we hypothesize that the pattern of NR1 overexpression induced by the AAV 1/2 vector, in particular high levels of NR1 expression in the interneurons both before and after kainate, likely influenced seizure phenotypes in this study. It is unclear how a transgene alone can alter the expression pattern of a given AAV vector. However different expression cassettes have been suggested to influence the conformation of virion capsid proteins, which could affect their interactions with cell-surface receptors (Franich et al., 2008; Mastakov et al., 2002).

Kainate-induced increases in the endogenous NR1 expression were seen in this study following NR1 knockdown and in control, eGFP injected rats. In support, similar increases in neuronal NR1 expression have been reported in human and animal epileptic tissue (Mathern et al., 1998; Rafiki et al., 1998). There is evidence to suggest that such changes may help alleviate neuronal damage, including in the kainate model (Lekishvili et al., 2006; Ogita et al., 2003). In contrast, when loss of NR1 expression occurs in seizures, it appears secondary to neuronal loss (Bayer et al., 1995), and our findings are consistent.

Positive influences on fear memory and neurogenesis observed by us with NR1 overexpression hint at the dichotomous function of NMDARs. Our observations were made in mature rats – approximately 6 months of age, transduced with NR1 at 4–6 weeks; and it is possible that the older age of animals at the time of testing influenced these results. We confirmed that transgene expression persisted over the entire experimental period (data not shown). Others have shown that NR1 expression declines with age in monkeys and humans, possibly contributing towards impairments in cognition (Gazzaley et al., 1996; Lu et al., 2004). In addition, NR1 expression levels are known to correlate with learning and memory in normal rats (Adams et al., 2001). Our strategy robustly increased NR1 expression predominantly in the dorsal hippocampus, and over an extended period of time (4–5 months). Other published studies into the effects of NR1 overexpression are few and mostly short-term (Adrover et al., 2003; Cheli et al., 2006; Cheli et al., 2002). However, NR2B transgenic mice, also characterized by increased levels of NR1 expression in the hippocampus, as well as mice with increased trafficking of NMDARs to neuronal surface, display superior cognition (Tang et al., 1999; Wong et al., 2002). A longer delay between vector transduction and behavioral testing in our study may have allowed a number of neuronal adaptations to occur. Further work is required to explore what adaptive changes develop over time, and if NR1 overexpression in younger rats and over a shorter time period could also achieve improvements in memory.

We did not examine in this study how our AAV-mediated strategies affected the surface and synaptic expression of the NR1 protein, and how this influenced NMDAR numbers,

trafficking and activity. Although we cannot comment what changes occurred in receptor stoichiometry, distribution and electrophysiological properties, our results indicate that enhanced NR1 expression may positively modulate NMDAR function. We believe, our findings provide a useful platform to closer examine molecular events that occur with NR1 knockdown and overexpression.

Experimental Methods

Plasmids and AAV vectors

Four siRNA sequences were designed to target rat *NR1* cDNA (GenBank accession number: X63255) (Table 1) using the siRNA Target Finder and Design Algorithm (Ambion, Austin, TX). Plasmid constructs to either overexpress or knock down the NR1 protein *in vitro* or *in vivo* were generated as described previously (Kalev-Zylinska and During, 2007). Briefly, to overexpress *NR1*, mouse *NR1* sequences (3031 bp; GenBank accession number D10028) were cloned into the AAV plasmid containing the cytomegalovirus early enhancer/chicken β -actin (CAG) promoter, the modified woodchuck posttranscriptional regulatory element (WPRE2), the shortened bovine growth hormone polyadenosine signal (spolyA), and the AAV 2 inverted terminal repeats (ITRs). The same AAV backbone but carrying the eGFP protein (Clontech, Palo Alto, CA) was used as control in this study. To knock down NR1, shNR1 sequences targeting *NR1* transcripts (at position 1561–1582 nt in the *NR1* cDNA; sense sequence: 5'-AAUGAGCGUGCGCAGUACAUA-3') were placed under the U6 promoter (p*Silencer* 1.0-U6 [Ambion]). The shNR1 structure also contained a nano-loop and a string of six T residues to provide a transcription termination site, as described (Cao et al., 2004). The U6-shNR1 cassette was cloned back-to-back with the CAG promoter driving dYFP, as a marker of expression. The backbone also contained the WPRE, polyA and the ITRs (Fitzsimons et al., 2002). Specificity of the shNR1 knockdown effects was validated previously using the shdYFP control (Kalev-Zylinska and During, 2007).

The DNA cassettes (containing either shNR1-dYFP, NR1 or eGFP) were packaged into recombinant AAV 1/2 vectors according to standard procedures (Hauck et al., 2003), purified using heparin affinity columns (Sigma, St Louis, MO), and titered as described (Clark et al., 1999).

Cell culture and transfections

Human embryonic kidney (HEK) 293 cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) (both from Life Technologies, Rockville, MD). Transient transfections with the DNA constructs were performed using FuGene 6 (Roche, Indianapolis, IN), according to manufacturer's instructions. For knockdown experiments, the required amount of siNR1 and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were diluted in Opti-MEM I (GibcoBRL, Gaithersburg, MD), and effects examined 72 h later. HEK293 cell line stably expressing the NR1 protein was generated by co-transfecting the NR1- and puromycin-resistance sequences, using a standard calcium phosphate precipitation method (Jordan and Wurm, 2004). Stable colonies were selected in the presence of puromycin and MK-801 (both from Sigma).

Fluorescence-activated cell sorting (FACS)

After dissociation with trypsin, HEK293 cells were washed several times and suspended in ice-cold phosphate buffered saline (PBS) supplemented with 5% FCS. FACS analysis was performed on a FACS Vantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ), based on forward and side scatter characteristics, and the fluorescence of dYFP or eGFP.

Experimental animals

Male Wistar rats (initially 190–240 g at 4–6 weeks of age) were used, housed in pairs in an animal facility under standard conditions ($23 \pm 1^\circ\text{C}$, $50 \pm 5\%$ humidity, 12 h light/dark cycle). Rats had free access to food (dry chow) and water. Behavioral testing was conducted during the dark phase of the light cycle, preceded by 7 days of animal handling. All procedures were approved by the Animal Ethics Committee of The University of Auckland, and performed in conformity with the institutional guidelines.

Surgical procedures

To infuse AAV vectors, rats were anesthetized with sodium pentobarbitone 60 mg/kg i.p. and 50 μl of 0.25% marcaine subcutaneously. Animal head was stabilized using Kopf (Tujunga, CA) stereotactic frames, and 3 μl of AAV 1/2 vector (3×10^9 genomic particles; titer matched for all vectors) was injected into the dorsal hippocampus bilaterally (for the kainate study) or unilaterally, on the right side of the brain (for fear memory and neurogenesis testing). The following coordinates (mm from bregma, horizontal skull) were used: anterior-posterior -3.6 , medio-lateral ± 2.0 and dorso-ventral -4.1 mm from the skull surface. Vector infusion was controlled by a microinfusion pump (World Precision Instruments, Hertfordshire, UK) at a rate of 66 nl/min.

Six weeks later, cannulae and electrodes were surgically implanted to administer kainate and to monitor EEG, respectively. The procedure was performed under stereotactic guidance on animals anesthetized as above, and essentially as described before (Vezzani et al., 2002). Bipolar, nichrome wire insulated electrodes (MS333/2B, Plastics One, Roanoke, VA) were implanted into the left hippocampus at the following coordinates: anterior-posterior -3.5 mm, medio-lateral $+2.5$ mm, dorso-ventral -3.6 mm. Guide cannula (22 gauge, 12 mm in length) was positioned above the dura through a skull hole on the right (anterior-posterior -3.5 mm, medio-lateral -2.5 mm), and filled with a 28 gauge stopper wire. Skull screws were inserted, one over the left parietal cortex and another over the nasal sinus. Implants were covered by a cap prepared from the tip of a 15 ml Falcon tube (Becton Dickinson) and secured to the skull with acrylic, cold-curing dental cement (Vertex, The Netherlands).

Behavioral testing

The seizure study was conducted 7–10 days after surgical implantation of electrodes and cannulae, according to a paradigm described previously (Vezzani et al., 2002). Rats were acclimatized in a plexiglas cage and the baseline EEG was recorded for 15 min using the EEG Amplifier and Data Recording System, model 79E (Grass Instruments Company, Quincy, MA). Kainate (OPIKA-1TM Kainic Acid [Ocean Produce International, Nova Scotia, Canada]) was injected (40 ng) into the dorsal hippocampus through the previously implanted cannula without animal anesthesia. EEG recordings were made continuously for 120 min after kainate administration. Traces were analyzed by an investigator blinded to the identity of the rats. Parameters measured included the latency to the first seizure (seizure onset), the number of seizures and their duration in the 2 h of EEG recordings. In addition, animal behavior was recorded on video throughout the 2 h observation period. Tapes were analyzed blindly for a full range of stereotypical seizure behaviors, according to the Racine rating scale (Racine, 1972). Briefly, stages were scored as follows: no change (Stage 0), immobility and staring (Stage 1), head nodding (Stage 2), forelimb clonus (Stage 3), rearing (Stage 4), generalized convulsions with loss of posture and falling (Stage 5). Five days after kainate administration, rats were killed and brains collected for tissue analysis.

Passive Avoidance testing was conducted 12 weeks after AAV vector infusion as described previously (Luttgen et al., 2005), with modifications (Kalev-Zylinska and During, 2007). In addition, electrical current delivered through the grid in this study was of 1 milliamp

amplitude and lasted 3 s. Memory retention was tested 24 h after training for a max of 600 s, and then on days 3 and 7 for rats that did not enter into the dark in prior sessions.

BrdU labeling

Rats were injected with 5-bromo-2-deoxyuridine (BrdU; Sigma) 50 mg/kg i.p. four times daily for 3 days, beginning 7 days after the last Passive Avoidance retention test (n = 10 per group). The BrdU was dissolved in sterile 0.9% NaCl solution containing 0.007 N NaOH. Half of the animals injected (n = 5 per group) were killed 24 h later to assess numbers of cells generated in the subgranular zone of the dentate gyrus. The remaining rats (n = 5 per group) were killed after further 4 weeks to determine a proportion of BrdU-labeled cells that survived and acquired the NeuN (Neuronal Nuclei) differentiation antigen.

Animals were killed by an overdose of sodium pentobarbitone i.p. Fresh brains were frozen on dry ice and stored at -80°C . Otherwise rats were perfused transcardially, and brains post-fixed and cryoprotected according to standard procedures (Young et al., 1999). Coronal sections of the hippocampus (16, 20 or 40 μm) were cut using Leica CM3050 freezing microtome (Leica Microsystems, Wetzlar, Germany).

Semi-quantitative Western blotting

Total protein was extracted from cultured HEK293 cells or from the excised hippocampal tissue using lysis buffer (50 mM Tris-HCl pH 7.4, 2mM EDTA, 0.05% Triton-X with complete protease inhibitor [Roche]) and sonicated on ice. Insoluble material was removed by centrifugation at 14,000 g for 10 min. Protein concentration was determined by colorimetric Bio-Rad Protein Assay (Hercules, CA), calibrated to bovine serum albumin.

Proteins (20–30 μg per lane) were separated by SDS/PAGE (sodium dodecyl polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham, Piscataway, NJ) using a semi-dry transfer cell module (Bio-Rad). Membranes were blocked for 3 h in a buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.2% Tween20) containing 5% non-fat dried milk powder, and then incubated overnight in a modified blocking buffer (1% milk powder) with a primary antibody: anti- NR1 (1:200), NR2A (1:2000) or NR2B (1:500) (all from Millipore, Billerica, MA). After washing, immunoreactive proteins were detected using horseradish-peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and the ECL detection system (NEN Life Sciences, Boston, MA).

To enable semi-quantitative analysis of changes in protein expression, blots were stripped (62.5 mM Tris pH 6.7, 2% SDS, 100 mM β -mercaptoethanol; 15 min at 55°C) and re-probed using an anti- β -actin antibody (1:5000; Abcam, Cambridge, UK) in 1% milk powder. Chemiluminescence signals were developed on a FujiFilm LAS-3000 phosphorimager. The Fuji Image MultiGauge software (Life Science, Stamford, CT), associated with the imaging station, was used to conduct semi-quantitative analysis of band densities. According to manufacturer's instructions, the densitometric values were first corrected for background and then normalized against β -actin. Serial dilutions of protein samples were used to construct calibration curves to ensure a linear relationship between band densities and the protein amounts loaded. This confirmed that the threefold changes in the expression of NR1, NR2A and NR2B proteins were within the linear sensitivity range.

Laser microdissection and RNA extraction

Fresh frozen brain sections (20 μm) were mounted onto slides, air-dried on ice for 20 s, fixed in ice-cold 70% ethanol for 1 min, and stored at -80°C . Prior to use, sections were kept on dry ice, incubated in 75, 96 and 100 % pre-cooled ethanol, 2 min each, and stained

in 0.5% cresyl violet solution for 1 min. Slides were dipped in 75 and 100 % ethanol and taken immediately for laser capture. All solutions were made in the diethylpyrocarbonate- (DEPC-) treated water.

Laser microdissection and pressure catapulting (LMPC) was carried out using PALM MicroLaser System (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) featuring a 337 nm nitrogen laser connected to Zeiss (MicroImaging GmbH, Germany) Axiovert 200 inverted microscope. System control was provided by the PALM RoboSoftware. Granule cell layers of the dentate gyrus were outlined, cut and pulsed with laser. Catapulted tissue fragments were collected directly in a cap of a microfuge tube placed above the slide, directly over the laser impact spots. Granule cell layers were dissected bilaterally from 8 sections per each animal into the same cap.

Immediately after LMPC, samples were put on dry ice and taken to extract RNA using an RNAqueous Micro Kit (Ambion), strictly according to manufacturer's instructions. RNA was eluted in 20 µl of Elution Solution preheated to 95 °C. Samples were treated with DNase I and RNA quantified using RiboGreen assay (Molecular Probes, Eugene, OR).

Reverse transcription and polymerase chain reaction (RT-PCR)

First-strand cDNA was generated using SuperSript III First-Strand Synthesis System (Invitrogen) with oligo(dT)₂₀ primers according to a procedure recommended by the manufacturer. Real-time RT-PCR was carried out on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), equipped with version 2.3 software. Primers were designed with the Express software (Applied Biosystems) against rat *NR1*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and *18S ribosomal RNA (18S rRNA)* mRNAs, with primer sequences as follows: *NR1*: 5'-AAT GAC CCC AGG CTC AGA AAC -3' (forward [fw]), 5'-CCA CGG AGC TCT GCT TTA CAG -3' (reverse [rev]); *Gapdh*: 5'-ACA TGC CGC CTG GAG AAA -3' (fw), 5'-CTC GGC CGC CTG CTT -3' (rev); and *18S rRNA*: 5'-GAT CCA TTG GAG GGC AAG TCT -3' (fw), 5'-CCA AGA TCC AAC TAC GAG CTT TTT -3' (rev). The 20 µl reaction was set up using *Power SYBR Green PCR Master Mix* (Applied Biosystems) with added SuperMix-UDG with ROX (Invitrogen), and the following thermal cycling profile: 50 °C 2 min, 95 °C 10 min, 40 cycles of amplification (95 °C 15 s, 60 °C 1 min). Each sample was tested in triplicate in three independent experiments. Amplification of a single product for each primer set was confirmed by a dissociation curve analysis. The relative gene expression was determined using a comparative 2-DeltaDelta-Cycle-threshold method, relative to the housekeeping gene. Data are presented as a fold difference of target gene transcripts in shNR1- and NR1-injected brains compared to the eGFP control.

Tissue labeling and stains

Single immuno- cyto- and histo- chemistry were performed as described (Young et al., 1999). The following primary antibodies were used: anti- NR1 (1:200), NR2A (1:500), NR2B (1:300) (Millipore), GFP (1:20,000; Abcam), BrdU (1:500; Accurate, Westbury, NY). Double immunofluorescence for BrdU and NeuN was performed sequentially using anti-BrdU antibody (as above) and a goat anti-rat-Cy3-labeled secondary antibody (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA), followed by a mouse monoclonal antibody against NeuN (1:1000; Millipore) and an anti-mouse-Cy5-labeled secondary antibody (1:250; Jackson ImmunoResearch).

Fluoro-Jade B staining was performed on 40 µm brain sections as described (Schmued and Hopkins, 2000). Six sections spaced 200 µm apart were analyzed for each animal. Tissue was examined using an Olympus AX70 microscope under FITC filter. Grading of damage

was performed by an investigator blinded to the experimental group. The extent of damage was scored as confined to the site of kainate injection only, present only in CA3 or seen both in CA3 and CA1.

Imaging

Low power images (where two sides of the hippocampus are shown) were taken using a DC200 digital camera and supporting software (Leica) connected to an MZFLIII stereomicroscope (Leica). Otherwise, an Olympus (Tokyo, Japan) AX70 microscope and CX9000 camera (MBF Bioscience, Williston, VT) with associated IQbase software were used. The Olympus microscope was also employed to count BrdU-positive cells in the subgranular zone of the dentate gyrus. The co-expression of BrdU and NeuN was analyzed under a TCS SP2 confocal laser scanning microscope (Leica). Excitation by Helium-Neon laser lines of 543 nm and 633 nm were used to image Cy3 and Cy5, respectively with the emission of 555–700 nm for Cy3, and 650–750 nm for Cy5. To image dYFP or eGFP, the 514 nm line from the Argon laser was used with the emission range of 525–650 nm. For each animal, BrdU counts were performed in six 40 μ m sections spaced 200 μ m apart.

Statistical analysis

Data are shown as the mean $\pm$ standard error of the mean (SEM). The SPSS 13 (Chicago, IL) and MedCalc (Mariakerke, Belgium) software packages were used to perform statistical analysis. Unless indicated otherwise, statistical significance of differences between multiple groups was assessed by one-way ANOVA. Dunnett *post-hoc* test was employed when comparing treatment groups against the control, and Scheffé *post-hoc* test when looking for differences between groups. In addition, to analyze results from the Passive Avoidance test, a Kaplan-Meier survival curve of time that rats took to enter the chamber was produced for each treatment group, and their difference tested using log rank test. The median time to enter the chamber, using the data from days 1, 3 and 7, i.e. until all rats had entered into the dark was compared using Kruskal-Wallis analysis of variance. The Kruskal-Wallis method allowed taking into account a varied time scale, as days have gone past between trials. *P* values less than 0.05 were considered statistically significant.

Acknowledgments

We acknowledge the help of Stephen Edgar with flow cytometry, Dr Jeff Keelan with laser microdissection, Jacqueline Ross with confocal microscopy, and members of the Crosier Laboratory for allowing access to the stereomicroscope. Sarah Borrie and Elizabeth Steinberg helped with tissue processing. Nicholas Franich advised on real-time RT-PCR, and Joanna Stewart on statistical analysis. The work was funded by the New Zealand New Economy Research Fund - Foundation for Research Science and Technology (UOAX0212) and the National Institute of Health (RO1 NS44576).

References

- Aarts MM, Tymianski M. Molecular mechanisms underlying specificity of excitotoxic signaling in neurons. *Curr Mol Med*. 2004; 4(2):137–47. [PubMed: 15032710]
- Adams MM, Smith TD, Moga D, Gallagher M, Wang Y, Wolfe BB, Rapp PR, Morrison JH. Hippocampal dependent learning ability correlates with N-methyl-D-aspartate (NMDA) receptor levels in CA3 neurons of young and aged rats. *J Comp Neurol*. 2001; 432(2):230–43. [PubMed: 11241388]
- Adrover MF, Guyot-Revol V, Cheli VT, Blanco C, Vidal R, Alche L, Kornisiuk E, Epstein AL, Jerusalinsky D. Hippocampal infection with HSV-1-derived vectors expressing an NMDAR1 antisense modifies behavior. *Genes Brain Behav*. 2003; 2(2):103–13. [PubMed: 12884967]
- Bayer TA, Wiestler OD, Wolf HK. Hippocampal loss of N-methyl-D-aspartate receptor subunit 1 mRNA in chronic temporal lobe epilepsy. *Acta Neuropathol (Berl)*. 1995; 89(5):446–50. [PubMed: 7618442]

- Ben-Ari Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience*. 1985; 14(2):375–403. [PubMed: 2859548]
- Bouilleret V, Ridoux V, Depaulis A, Marescaux C, Nehlig A, Le Gal La Salle G. Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. *Neuroscience*. 1999; 89(3):717–29. [PubMed: 10199607]
- Brandt C, Potschka H, Loscher W, Ebert U. N-methyl-D-aspartate receptor blockade after status epilepticus protects against limbic brain damage but not against epilepsy in the kainate model of temporal lobe epilepsy. *Neuroscience*. 2003; 118(3):727–40. [PubMed: 12710980]
- Cao L, Jiao X, Zuzga DS, Liu Y, Fong DM, Young D, During MJ. VEGF links hippocampal activity with neurogenesis, learning and memory. *Nat Genet*. 2004; 36(8):827–35. [PubMed: 15258583]
- Chapman AG, Woodburn VL, Woodruff GN, Meldrum BS. Anticonvulsant effect of reduced NMDA receptor expression in audiogenic DBA/2 mice. *Epilepsy Res*. 1996; 26(1):25–35. [PubMed: 8985683]
- Cheli V, Adrover M, Blanco C, Ferrari C, Cornea A, Pitossi F, Epstein AL, Jerusalinsky D. Knocking-down the NMDAR1 subunit in a limited amount of neurons in the rat hippocampus impairs learning. *J Neurochem*. 2006; 97(Suppl 1):68–73. [PubMed: 16635252]
- Cheli VT, Adrover MF, Blanco C, Rial Verde E, Guyot-Revol V, Vidal R, Martin E, Alche L, Sanchez G, Acerbo M, et al. Gene transfer of NMDAR1 subunit sequences to the rat CNS using herpes simplex virus vectors interfered with habituation. *Cell Mol Neurobiol*. 2002; 22(3):303–14. [PubMed: 12469872]
- Chittajallu R, Vignes M, Dev KK, Barnes JM, Collingridge GL, Henley JM. Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. *Nature*. 1996; 379(6560):78–81. [PubMed: 8538745]
- Christie JM, Schoppa NE, Westbrook GL. Tufted cell dendrodendritic inhibition in the olfactory bulb is dependent on NMDA receptor activity. *J Neurophysiol*. 2001; 85(1):169–73. [PubMed: 11152717]
- Ciani E, Rizzi S, Paulsen RE, Contestabile A. Chronic pre-explant blockade of the NMDA receptor affects survival of cerebellar granule cells explanted in vitro. *Brain Res Dev Brain Res*. 1997; 99(1):112–7.
- Clark KR, Sferra TJ, Lo W, Qu G, Chen R, Johnson PR. Gene transfer into the CNS using recombinant adeno-associated virus: analysis of vector DNA forms resulting in sustained expression. *J Drug Target*. 1999; 7(4):269–83. [PubMed: 10682906]
- Clifford DB, Olney JW, Benz AM, Fuller TA, Zorumski CF. Ketamine, phencyclidine, and MK-801 protect against kainic acid-induced seizure-related brain damage. *Epilepsia*. 1990; 31(4):382–90. [PubMed: 2196169]
- Dimagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci*. 2003; 26(5):248–54. [PubMed: 12744841]
- During MJ, Spencer DD. Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *Lancet*. 1993; 341(8861):1607–10. [PubMed: 8099987]
- Fitzsimons HL, Bland RJ, During MJ. Promoters and regulatory elements that improve adeno-associated virus transgene expression in the brain. *Methods*. 2002; 28(2):227–36. [PubMed: 12413421]
- Franich NR, Fitzsimons HL, Fong DM, Klugmann M, During MJ, Young D. AAV vector-mediated RNAi of mutant huntingtin expression is neuroprotective in a novel genetic rat model of Huntington's disease. *Mol Ther*. 2008; 16(5):947–56. [PubMed: 18388917]
- Gazzaley AH, Siegel SJ, Kordower JH, Mufson EJ, Morrison JH. Circuit-specific alterations of N-methyl-D-aspartate receptor subunit 1 in the dentate gyrus of aged monkeys. *Proc Natl Acad Sci U S A*. 1996; 93(7):3121–5. [PubMed: 8610179]
- Haberman R, Criswell H, Snowdy S, Ming Z, Breese G, Samulski R, McCown T. Therapeutic liabilities of in vivo viral vector tropism: adeno-associated virus vectors, NMDAR1 antisense, and focal seizure sensitivity. *Mol Ther*. 2002; 6(4):495–500. [PubMed: 12377191]
- Hauck B, Chen L, Xiao W. Generation and characterization of chimeric recombinant AAV vectors. *Mol Ther*. 2003; 7(3):419–25. [PubMed: 12668138]

- Huerta PT, Sun LD, Wilson MA, Tonegawa S. Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons. *Neuron*. 2000; 25(2):473–80. [PubMed: 10719900]
- Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovská V, Turski L, Olney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science*. 1999; 283(5398):70–4. [PubMed: 9872743]
- Ikonomidou C, Stefovská V, Turski L. Neuronal death enhanced by N-methyl-D-aspartate antagonists. *Proc Natl Acad Sci U S A*. 2000; 97(23):12885–90. [PubMed: 11058158]
- Jordan M, Wurm F. Transfection of adherent and suspended cells by calcium phosphate. *Methods*. 2004; 33(2):136–43. [PubMed: 15121168]
- Kalev-Zylinska ML, During MJ. Paradoxical facilitatory effect of low-dose alcohol consumption on memory mediated by NMDA receptors. *J Neurosci*. 2007; 27(39):10456–67. [PubMed: 17898217]
- Kato H, Liu Y, Araki T, Kogure K. MK-801, but not anisomycin, inhibits the induction of tolerance to ischemia in the gerbil hippocampus. *Neurosci Lett*. 1992; 139(1):118–21. [PubMed: 1407677]
- Kaufer D, Ogle WO, Pincus ZS, Clark KL, Nicholas AC, Dinkel KM, Dumas TC, Ferguson D, Lee AL, Winters MA, et al. Restructuring the neuronal stress response with anti-glucocorticoid gene delivery. *Nat Neurosci*. 2004; 7(9):947–53. [PubMed: 15300253]
- Lees KR, Asplund K, Carolei A, Davis SM, Diener HC, Kaste M, Orgogozo JM, Whitehead J. Glycine antagonist (gavestinel) in neuroprotection (GAIN International) in patients with acute stroke: a randomised controlled trial. GAIN International Investigators. *Lancet*. 2000; 355(9219):1949–54. [PubMed: 10859040]
- Lekishvili T, Hesketh S, Brazier MW, Brown DR. Mouse galectin-1 inhibits the toxicity of glutamate by modifying NR1 NMDA receptor expression. *Eur J Neurosci*. 2006; 24(11):3017–25. [PubMed: 17156363]
- Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA. Gene regulation and DNA damage in the ageing human brain. *Nature*. 2004; 429(6994):883–91. [PubMed: 15190254]
- Luttgen M, Elvander E, Madjid N, Ogren SO. Analysis of the role of 5-HT1A receptors in spatial and aversive learning in the rat. *Neuropharmacology*. 2005; 48(6):830–52. [PubMed: 15829255]
- Maren S. Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. *Trends Neurosci*. 1999; 22(12):561–7. [PubMed: 10542437]
- Mastakov MY, Baer K, Symes CW, Leichtlein CB, Kotin RM, During MJ. Immunological aspects of recombinant adeno-associated virus delivery to the mammalian brain. *J Virol*. 2002; 76(16):8446–54. [PubMed: 12134047]
- Mathern GW, Pretorius JK, Mendoza D, Lozada A, Leite JP, Chimelli L, Fried I, Sakamoto AC, Assirati JA, Adelson PD. Increased hippocampal AMPA and NMDA receptor subunit immunoreactivity in temporal lobe epilepsy patients. *J Neuropathol Exp Neurol*. 1998; 57(6):615–34. [PubMed: 9630240]
- McHugh TJ, Jones MW, Quinn JJ, Balthasar N, Coppari R, Elmquist JK, Lowell BB, Fanselow MS, Wilson MA, Tonegawa S. Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network. *Science*. 2007; 317(5834):94–9. [PubMed: 17556551]
- Melik E, Babar E, Ozen E, Ozgunen T. Hypofunction of the dorsal hippocampal NMDA receptors impairs retrieval of memory to partially presented foreground context in a single-trial fear conditioning in rats. *Eur Neuropsychopharmacol*. 2006; 16(4):241–7. [PubMed: 16176870]
- Morris GF, Bullock R, Marshall SB, Marmarou A, Maas A, Marshall LF. Failure of the competitive N-methyl-D-aspartate antagonist Selfotel (CGS 19755) in the treatment of severe head injury: results of two phase III clinical trials. The Selfotel Investigators. *J Neurosurg*. 1999; 91(5):737–43. [PubMed: 10541229]
- Nakazawa K, Quirk MC, Chitwood RA, Watanabe M, Yeckel MF, Sun LD, Kato A, Carr CA, Johnston D, Wilson MA, et al. Requirement for hippocampal CA3 NMDA receptors in associative memory recall. *Science*. 2002; 297(5579):211–8. [PubMed: 12040087]
- Ogita K, Okuda H, Yamamoto Y, Nishiyama N, Yoneda Y. In vivo neuroprotective role of NMDA receptors against kainate-induced excitotoxicity in murine hippocampal pyramidal neurons. *J Neurochem*. 2003; 85(5):1336–46. [PubMed: 12753091]
- Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol*. 1972; 32(3):281–94. [PubMed: 4110397]

- Rafiki A, Ben-Ari Y, Khrestchatisky M, Represa A. Long-lasting enhanced expression in the rat hippocampus of NMDAR1 splice variants in a kainate model of epilepsy. *Eur J Neurosci.* 1998; 10(2):497–507. [PubMed: 9749712]
- Rampon C, Tang YP, Goodhouse J, Shimizu E, Kyin M, Tsien JZ. Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. *Nat Neurosci.* 2000; 3(3):238–44. [PubMed: 10700255]
- Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res.* 2000; 874(2):123–30. [PubMed: 10960596]
- Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, Liu G, Tsien JZ. Genetic enhancement of learning and memory in mice. *Nature.* 1999; 401(6748):63–9. [PubMed: 10485705]
- Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH. NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. *Nature.* 2006; 442(7105):929–33. [PubMed: 16906136]
- Tsien JZ, Huerta PT, Tonegawa S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell.* 1996; 87(7):1327–38. [PubMed: 8980238]
- Vezzani A, Michalkiewicz M, Michalkiewicz T, Moneta D, Ravizza T, Richichi C, Aliprandi M, Mule F, Pirona L, Gobbi M, et al. Seizure susceptibility and epileptogenesis are decreased in transgenic rats overexpressing neuropeptide Y. *Neuroscience.* 2002; 110(2):237–43. [PubMed: 11958866]
- Wang Q, Yu S, Simonyi A, Sun GY, Sun AY. Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol Neurobiol.* 2005; 31(1–3):3–16. [PubMed: 15953808]
- White HS, Smith MD, Wilcox KS. Mechanisms of action of antiepileptic drugs. *Int Rev Neurobiol.* 2007; 81:85–110. [PubMed: 17433919]
- Wong RW, Setou M, Teng J, Takei Y, Hirokawa N. Overexpression of motor protein KIF17 enhances spatial and working memory in transgenic mice. *Proc Natl Acad Sci U S A.* 2002; 99(22):14500–5. [PubMed: 12391294]
- Young D, Lawlor PA, Leone P, Dragunow M, During MJ. Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat Med.* 1999; 5(4):448–53. [PubMed: 10202938]
- Zapata A, Capdevila JL, Tarrason G, Adan J, Martinez JM, Piulats J, Trullas R. Effects of NMDA-R1 antisense oligodeoxynucleotide administration: behavioral and radioligand binding studies. *Brain Res.* 1997; 745(1–2):114–20. [PubMed: 9037399]

Fig. 1.

Characterization of NR1 knockdown *in vitro*. (A-C) Effects of siNR1- 1, 2 and 3 are shown in comparison to a control siRNA sequence targeting dYFP (sidYFP). The effects of the fourth sequence, siNR1-4 were identical to siNR1-1 i.e. both were ineffective in reducing NR1 expression (not shown). (A) NR1 immunocytochemistry on HEK293 cells co-transfected with the *NR1* cDNA and siRNAs as indicated. The siNR1-2 produced mild, and siNR1-3 moderate knockdown of NR1 expression. (B) Representative levels of NR1 expression by Western blot in extracts from HEK293 cells transfected as above. (C) Quantitative analysis of three independent immunoblots as in B, expressed as a fold reduction relative to the untreated control. There was no non-specific off-target knockdown of NR1 by the sidYFP control. Bars represent mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.005$ (one-way ANOVA). (D) Validation of a bicistronic, pAM-shNR1-3-dYFP construct, designed for the use *in vivo*. HEK293 cells with stable NR1 expression were transiently transfected with the pAM-shNR1-3-dYFP. Cells expressing dYFP were gated on by FACS (R3, R2 and M2 gates are shown), and 100,000 cells were sorted. SSC, side scatter; FSC, forward scatter

characteristics. (E) Representative NR1 expression in lysates from sorted cells, compared to control transfections with a non-silencing, eGFP expressing construct. β -actin served as a loading control. Immunoblot experiments were repeated three times, yielding similar results. (F–I) In transient co-transfection experiments on HEK293 cells, siNR1-3 did not reduce expression of NR2A nor NR2B when examined by immunocytochemistry (F, G) and semi-quantitative immunoblots (H, I) in three independent experiments. Bars represent mean $\pm$ SEM; ns, non-significant (one-way ANOVA). Scale bars in A and G, 200 μ m.

Fig. 2.

Knockdown and overexpression of NR1 in rat hippocampus. (A) Representative images of the hippocampal expression of eGFP or NR1 6 weeks after unilaterally injecting eGFP-, shNR1- or NR1- carrying AAV vectors. Expression is shown at two different hippocampal levels in the dorso-ventral axis; injected sides are indicated by arrowheads. (B,C) Higher magnification views of NR1 knockdown (kd; B) and overexpression (oe; C) within the regions of dentate gyrus (DG) and CA3, shown in comparison to the uninjected, contralateral side. In C, strong NR1 expression in the principal cells and interneurons is indicated by arrowheads and arrows, respectively. (D) Immunoblots showing expression of the NR1, NR2B and NR2A proteins in the NR1-injected hippocampi, compared with the eGFP-injected control. β -actin served as a loading control. Experiments were performed three times, yielding similar results. (E) Laser microdissection and pressure catapulting

(LMPC) was performed on fresh frozen hippocampal sections, counter-stained with cresyl violet. Granule cell layers were marked, cut, fired by laser and catapulted, as indicated by arrows in a–d panels, respectively. (F) Relative levels of *NR1* transcripts determined by real-time RT-PCR in specimens excised by LMPC, expressed as a fold change relative to eGFP control. Data are mean $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$ (one-way ANOVA). Scale bars: in A, 1 mm; in B, C and E, 100 μ m.

Fig. 3. Characteristics of EEG seizures induced by kainate. (A) Timeline of the kainate-induced seizure experiment; $n = 6$ rats per group. KA, kainate. (B) Schematic of brain manipulations. 40 ng KA was infused directly into the hippocampus through a previously inserted cannula. EEG and behavioral monitoring continued over 120 min. (C) Table containing quantification of EEG seizures induced by kainate, analyzed as described in Materials and Methods. Data are mean ± SEM. * $p < 0.05$; ** $p < 0.005$ (one-way ANOVA). Rats with the hippocampal NR1 knockdown displayed much less seizures, and at longer latencies. (D) Examples of EEG traces depicting seizure activity in eGFP-, NR1- and shNR1- injected rats. Time-frame that elapsed from the intrahippocampal administration of kainate is shown

above the traces. The majority of seizures in the NR1 overexpressing animals occurred between 61–120 min. EEG calibration: 1 mV/1 min.

Fig. 4. Behavioral characteristics of seizures induced by kainate. (A) Table containing quantification of seizure behaviors according to staging by Racine. Data are mean ± SEM, n = 6 per group. * $p < 0.05$ (one-way ANOVA); nr, not reached. Rats with NR1 overexpression displayed longer latencies to seizure stages II and III, and a few stage I only seizures were observed in NR1 knockdown rats. (B) Pie charts demonstrating frequency of Racine behaviors in eGFP-, NR1- and shNR1- injected rats shown as a mean percentage of total time in seizure for all rats within groups. (C) Effects of eGFP- (□), NR1- (○) and shNR1- (▲) injections on kainate-induced behavioral seizure scores during 120 min monitoring period at 15 min intervals. Data are mean ± SEM, n = 6 per group. * $p < 0.05$ (one-way ANOVA).

Fig. 5.

Neuronal damage after kainate. (A) Minimal (arrows) and maximal changes seen with Fluoro-Jade B in brains injected with eGFP, NR1 and shNR1 ($n = 6$ per group). Panels labeled 1 and 2 contain higher magnification views of their corresponding regions in proceeding panels. (B) Pie charts demonstrating the amount of Fluoro-Jade B (FJB) damage observed in hippocampi injected with eGFP, NR1 and shNR1. We arbitrarily scored the damage as localized to the injection site or CA3 only, or seen both in CA3 and CA1. Numbers of rats in each group is shown. In shNR1-injected rats, FJB damage was mostly confined to the site of kainate injection. A wider range of damage was seen associated with NR1 overexpression. (C) Typical H&E appearances of the kainate-injected hippocampi (inj hipp), together with representative images of CA3 and CA1 regions, in brains injected with eGFP, NR1 and shNR1. Insets contain higher magnification views of areas indicated by

arrowheads in their respective panels. Compared with the loss of nuclei in eGFP-injected controls, the presence of pyknotic nuclei dominated in CA1 in the NR1-injected brains (insets, white arrows). Black arrows (insets) point to large vacuoles seen within the pyramidal cell nuclei in the shNR1-injected brains, mostly located in CA3. Scale bars in A and C, 50 μm .

Fig. 6. NR1 expression patterns before and after kainate. (A) Cells containing the NR1 knockdown cassette have been marked by the presence of dYFP. Robust expression of the knockdown construct occurred in the pyramidal neurons, mossy fibres and granule cells (arrows), and in occasional astrocytes (arrowhead), but it was not seen in the interneurons. (B) Sites of NR1 overexpression. Strong presence of NR1 is shown in pyramidal and granule cells (black arrows), interneurons (black arrowheads) and in the subgranular zone (white arrow). (C–F) Changes in NR1 expression induced by kainate and seizures. Regions with reduced NR1 expression corresponding with neuronal loss are shown in eGFP-injected hippocampi (C, arrows). In addition, in the eGFP- (D) and shNR1- (E) injected hippocampi, cells with

increased NR1 expression could be found amongst granule and pyramidal neurons (arrowheads). (F) In NR1-injected hippocampi, kainate seizures caused a reduction in NR1 expression in the principal neurons (arrows), but its expression remained strong in the interneurons (arrowheads) within all hippocampal regions (DG, CA1 and CA3). Scale bars, 100 μm .

Fig. 7.

NR1 expression and fear memory. (A) Design and timeline of the experiment that tested effects of the modulated NR1 expression on fear memory and neurogenesis. PA, Passive Avoidance; BrdU, 5-Bromo-2-deoxyuridine. Twelve rats per group were subjected to PA testing, of which n = 10 per group later received BrdU injections. The remaining n = 2 per group were killed at the end of the study to complete analysis of expression. (B) Times to enter dark chamber (step-through latency) during training (day 0). Data are mean $\pm$ SEM, n = 12 per group. There was no difference between groups (ns, non-significant; one-way ANOVA). (C) Absolute numbers of rats that entered dark chamber on days 1, 3 and 7. * $p < 0.05$, one-way ANOVA. (D) Kaplan-Meier curves demonstrating probability of entry into the dark on day 1, but taking into account all rats that did not enter at that time. NR1

rats took longest, and shNR1 rats shortest time to enter (log rank test $p = 0.01$; $n = 12$ per group). Similarly, comparing median times to enter for all days (Kruskal-Wallis test, censored for each group), there was an evidence of a difference ($p = 0.01$; $n = 12$ per group): again NR1 rats showed longest, and shNR1 shortest latencies.

Fig. 8. NR1 expression and neurogenesis. Representative images of BrdU immunohistochemistry and the numbers of BrdU positive (BrdU⁺) cells counted in the subgranular zones at 24 h (A,B respectively) and 4 weeks (C,D) after BrdU administration. In A and C, boxes mark regions that are shown magnified below. Arrowheads point to BrdU⁺ cells located in the subgranular zone. (B,D) Numbers of BrdU⁺ cells per subgranular zone of dentate gyrus per section are shown in relation to the transgene expression (eGFP, NR1 or shNR1). Bars represent mean $\pm$ SEM; n = 5 per group. * p < 0.05, ** p < 0.005 (one-way ANOVA). Knockdown of NR1 reduced proliferation of cells in the subgranular zone, and NR1 overexpression increased numbers of cells that survived at 4 weeks. (E) Confocal micrographs of BrdU (red) and NeuN (blue) staining, where newly generated neurons are double positive (BrdU⁺NeuN⁺). (F) Numbers of BrdU⁺NeuN⁺ cells per subgranular zone of dentate gyrus per section. There was no evidence of a difference in neuronal differentiation

between groups. Data are mean $\pm$ SEM; n = 5 per group. ns, non-significant (one-way ANOVA). Scale bars, 50 μ m.

Table 1

Sequences of siRNAs used in this study to target rat *NR1* transcripts.

siNR1 name	Sense sequence 5' to 3'	Target nucleotides in rat <i>NR1</i> cDNA (X63255)
siNR1-1	AAGCGGAACAUCUGUUAUGC	109 – 130
siNR1-2	AAUGGCUUCAUGGAAGAUCUG	2344 – 2365
siNR1-3	AAUAUGUACUGCGCACGCUCA	1561 – 1582
siNR1-4	AAUGUCUCCAGAUGUUGGUG	931 – 952