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Development of an inducible NMDA receptor stable cell line with an intracellular Ca^{2+} reporter

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Abstract

Cytotoxicity associated with NMDA receptor activation has impeded the establishment of cell lines expressing recombinant subtypes of this ligand-gated ion channel class. To circumvent this toxicity, we describe in this report the use of a potent inducible promoter in the construction of a cell line stably expressing the NR1a/NR2A subtype of the NMDA receptor. Western blot analysis using subunit selective antibodies revealed that NR2A subunits were constitutively expressed in this cell line, whereas expression of NR1a subunits was tightly regulated by tetracycline. Upon tetracycline removal, electrophysiological recordings using the patch clamp technique indicated the expression of functional receptors with biophysical and pharmacological properties corresponding to those expected of the NR1a/NR2A subtype. In addition, we utilized this cell line with the recombinant membrane targeted Ca^{2+} reporter, aequorin, in a functional assay of NMDA receptor activation. An evaluation of the coupling efficiency of NMDA receptor activation and aequorin response, as well as the pharmacological profile of this assay, illustrates the suitability of this cell line and the Ca^{2+} reporter assay to functionally identify novel NMDA receptor antagonists.

Keywords: NMDA receptor; Promoter inducible; Reporter gene assay; Aequorin

1. Introduction

NMDA receptors are ligand-gated cation channels which constitute a distinct class within the glutamate receptor family (Nakanishi, 1992; Hollmann and Heinemann, 1994). This NMDA receptor class has been the subject of particular interest in that these receptors are considered to play a key role in important neuronal processes such as synaptic plasticity and to be associated with certain neuropathophysiological disorders including acute and chronic neurodegeneration (Choï, 1987; Collingridge and Bliss, 1987). The pharmacology of the NMDA receptor is unique in that it requires activation by two co-agonists glutamate and glycine (Johnson and Ascher, 1987). In addition to competitive antagonists at each of these agonist binding sites, the NMDA receptor also possesses numerous modulatory sites, e.g., those that are sensitive to zinc, polyamines, proton, reducing agents, histamine, ethanol or can be blocked by open channel blockers (some of them reviewed by Sucher et al., 1996). Molecular cloning of the NMDA receptor has revealed the existence of several receptor subunits. The first described subunit, NR1 (ζ1 in mouse), is the product of a single gene that can in fact generate eight splice variants, NR1a, NR1b…NR1h (Sugihara et al., 1992; Hollmann et al., 1993). In addition, NR2 subunits constitute four homologous proteins derived from four different genes, namely NR2A, NR2B, NR2C and NR2D in rat and human, and ε1 to ε4 in mouse (Monyer et al., 1992; Ishii et al., 1993; Le Bourdellès et al., 1994). A new modulatory subunit, NR3A, has been recently described (Das et al., 1998). Although the subunit composition of native NMDA receptors remains to be elucidated, assembly of NR1 and NR2 subunits is necessary to constitute a fully functional receptor (Moriyoshi et al., 1991; Sugihara et al., 1992; Ishii et al., 1993). Using binary NR1/NR2 subunit combinations, it has been demonstrated that the nature of the NR2 subunit influences dramatically the biophysical and pharmacological properties of the resultant functional recombinant NMDA receptor (reviewed by Sucher et al., 1996). By this means, it has been possible to identify certain compounds that discriminate between

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different binary NR1/NR2 combinations. For example, ifenprodil potently blocks NMDA receptors containing the NR1/NR2B subtype but has almost no effect on other subtypes containing NR1 coexpressed with other NR2 subunits (Williams, 1995; Besnard et al., 1996).

The availability of cell lines that express defined subunit combinations of NMDA receptors would provide convenient in vitro cellular models to identify and characterize selective agonists and antagonists of native NMDA receptor subtypes. However, neurotoxicity is a well-characterized property of NMDA receptor activation, notably in neuronal cell culture models (Choï, 1987). Moreover, this phenomenon also arises upon sustained transient expression (> 24 h) of certain NR1/NR2 subunit combinations (Cik et al., 1993; Anegawa et al., 1995; Boeckman and Aizenman, 1996). In an attempt to overcome this toxicity, the conjugated use of NMDA receptor antagonists and a weakly inducible promoter (Priestley et al., 1995), as well as a heat shock-induced promoter (Uchino et al., 1997) to develop a stable cell line has recently been described.

In the present study, we chose to develop a cell line stably expressing the NR1a/NR2A combination as a model and in the construction of this cell line, we used a powerful inducible system repressed by tetracycline (Gössen and Bujard, 1992). The stringent control of NMDA receptor expression obtained with this system offers significant improvement over those described previously (Priestley et al., 1995; Uchino et al., 1997). In addition, we were able to assess the functional interaction of compounds with this receptor using aequorin as a Ca^{2+} reporter in an assay which is adaptable to functional analysis on a larger scale. Aequorin has previously been described as a reporter for a number of other receptors associated with Ca^{2+} flux (Shimomura, 1993). Apo-aequorin is a 21-kDa photoprotein which becomes active when linked to its cofactor, coelenterazine. When Ca^{2+} binds to aequorin a photon is produced upon oxidation of coelenterazine, and its rate of consumption is reported to be proportional to Ca^{2+} levels over the physiological concentration range (100 nM–100 μM; Brini et al., 1995). Use of these combined systems allowed us to (i) circumvent toxicity encountered when using a constitutive expression system, and (ii) develop a highly reproducible functional assay suitable to the study of a complex pharmacology associated with the NMDA receptor.

2. Materials and methods

2.1. Materials

Materials were obtained from the following sources AP5 (D-2-amino-5-phosphonopentanoic acid), ketamine from Sigma; CPP (*R*-3(2-carboxypiperazin-4-yl)propyl-phosphonate), 5,7-dichlorokynurenic acid, DNQX (6,7-dinitroquinoxaline-2,3-dione), HA966 ((+)-3*R*-3-amino-1-hydroxypyrridolin-2-one) from Tocris Cookson; dextromethorphan, dextrorphan from RBI; geneticin from Gibco BRL; CGS19755, eliprodil, ifenprodil, fluoxetine, L689.560, Memantine, MK801 (dizocilpine or (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) and phencyclidine from Synthelabo; zeocin from Invitrogen.

2.2. Plasmid constructions

pUHD15.1 and PUHG10.3 were obtained from Dr. Bujard, University of Heidelberg, Germany. pUHD15.1 encodes a chimeric tTA (tetracycline sensitive TransActivator) transactivator and pUHD10.3 bears a minimal promoter inducible in the presence of the tTA transactivator. A multicloning site (5′GCTAGCAAGCTTCTGCAGCTGC TTAAGGATATCGTCGACTAGTTAACGCTAGCGCGGCCGCTCTAGA-3′) was introduced in *Bam*HI and *Xba*I sites of pUHD10.3 and the plasmid was renamed pCTpl1. The rat cDNAs for NMDA receptors, NR1a, NR2A were a gift of Prof. S. Nakanishi, University of Kyoto (Moriyoshi et al., 1991). The NR1a cDNA was subcloned from pN60 into pCTpl1 using *Hin*dIII and *Eco*rV restriction sites keeping 265 bp of 5′ and 260 bp of 3′ non coding sequence and named pCT-NR1a. The plasmid p3Z-NR2A was constructed by subcloning the NR2A cDNA from pNR2A into *Not*I and *Xba*I restriction sites of pcDNA3Zeo (Invitrogen) leaving 79 bp of 5′ and 770 bp of 3′ non-coding sequence. p3′SS (kit LacSwitch, Stratagene) bearing hygromycin resistance was cut with *Bam*HI and *Hin*dIII, blunt ended and religated to produce pHygro, a pure hygromycin resistance vector.

Luciferase from pGEM-luc (Promega) was also inserted in the pCT-pl1 vector to measure the induction potential of the different cell lines established. mAequorin is a chimera (gift of Dr. Rizzuto, University of Padova) between SNAP25 (a neuronal protein recruited to the plasma membrane) and Aequorin inserted into the pcDNA-I mammalian expression vector (Marsault et al., 1997); this chimeric aequorin gave in our hands a higher luminescence than classic aequorins. Plasmids were purified by ion exchange chromatography (Qiagen).

2.3. Transfection procedures

HEK 293 cells (ATCC 1573; Human Embryonic Kidney) were used in these experiments and were seeded at 4×10^6 cells per 100 mm dish in Minimum Essential Medium (Gibco BRL) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). Transfections were performed according to the Chen and Okayama protocol (Chen and Okayama, 1987). Briefly, 20 μg of plasmid were precipitated with Ca^{2+} phosphate at pH 6.96 for a 100 mm dish at a 5:1 ratio of expression plasmid to resistance gene

plasmid. For stable cell line generation, cells contained in each 100 mm dish were dissociated two days after transfection and replated at 1/20 density. Cells were exposed the next day to selection medium containing Geneticin (1 mg/ml) for Neo resistance, hygromycin (100 μg/ml) for Hygro resistance, or Zeocin (100 μg/ml) for Zeocin resistance. The medium was replaced every two or three days over a period of two weeks. After this time, islets of cells were observed which were dissociated and grown separately as clones. Cells were maintained in medium supplemented with tetracycline (1 μg/ml) to inhibit NR1 expression during transfection with NR2 subunits and subsequent culture.

2.4. Luciferase assay

Luciferase activity was recorded in a Dynatech ML3000 Luminometer using luciferin from Promega according to the manufacturer's instructions.

2.5. Western blot

For the Western Blots, either 5 μg of membrane preparations or lysates of 20,000 cells were loaded on sodium dodecyl sulfate polyacrylamide gels (5–15% gradient). After migration proteins were transferred to Hybond ECL membrane (Amersham). Saturation and antibodies incubations were performed in 20 mM Tris–HCl pH 7.4 containing 140 mM NaCl and 5% dehydrated milk powder, and washing steps in 20 mM Tris–HCl pH 7.4 containing 140 mM NaCl and 0.05% Tween 20. Antibodies against NR1a (0.6 μg/ml) and NR2A/NR2B (1 μg/ml) NMDA receptor subunits were provided by Prof. F.A. Stephenson, University of London (Cik et al., 1993). Second antibody was a goat anti-rabbit immunoglobulin linked to peroxidase (Jackson). The ECL chemiluminescent reagent (Amersham) was used as a substrate following the manufacturer's instructions.

2.6. Electrophysiology

Whole-cell patch-clamp experiments were carried out on HEK293 (human embryonic kidney 293) cells that had been plated on glass coverslips coated with polylysine and then maintained 24–48 h in culture media containing 200 μM AP5 but no tetracycline. The extracellular medium was composed of 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose and 10 μM glycine (pH = 7.3 adjusted with NaOH). Pipettes pulled from borosilicate glass were filled with a solution containing 135 mM CsCl, 4 mM NaCl, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM Mg-ATP and 10 mM HEPES (pH = 7.3 with CsOH) and had a resistance of 2–5 MΩ. All the experiments were performed on isolated cells, at room temperature (20–24°). All data were acquired and analysed using the pClamp 6.0 software (Axon Instruments).

2.7. Aequorin assay

Cells transiently transfected with mAequorin (Marsault et al., 1997) were seeded in the presence of 0.5 mM ketamine (this use-dependent drug was the most suitable to protect the cells for 48 h) at 30,000 cells per well in a 96-well L-view Plate (Packard) for 24–48 h. Cells were then incubated with 5 μM h-coelenterazine (Molecular Probes) and 200 μM AP5 for 2 h (AP5 was used to prevent calcium entry during reconstitution and was easy to wash). Plates were briefly rinsed with Ringer solution containing 1 mM EDTA (without Mg^{2+}) and then incubated in 200 μl of Ringer solution (without Mg^{2+}) in the presence of antagonists prepared from aqueous or 1% (v/v) dimethyl sulfoxide solutions. Each well was read for 10 s in a multiwell luminometer (Dynatech) after injection of 50 μl of Ringer (without magnesium) plus 100 μM glutamate, 10 μM glycine, 100 μM spermine and 2 mM Ca^{2+}. This luminescent signal was integrated over 10 s to measure aequorin consumption following NMDA receptor activation.

Each data point represents the mean of three wells. IC_{50} values were determined by nonlinear regression analysis using the Origin 4.1 software (Microcal, MA), with 100% activity defined as that obtained in the absence of antagonist and 0% activity as that obtained upon preincubation with 200 μM AP5.

3. Results

3.1. Establishment of a transactivating cell line

HEK293 cells were transfected in the first instance with chimeric tetracycline tTA transactivator and Neomycin resistance plasmids. Upon selection with Geneticin, 100 clones were isolated which were subsequently screened by transient expression using a construct in which we had placed a luciferase reporter gene under the control of the tTA regulated inducible promoter. Luciferase level was then measured in the presence or absence of tetracycline. The clone showing the highest degree of induction of luciferase activity (approximately 100 fold) was chosen for further studies. Induction by the promoter system in this cell line proved to be stable for at least 20 passages, and thus this clone was used as the starting point for the subsequent development of the inducible cell lines described below.

3.2. Establishment of a stable cell line with inducible expression of the NMDA receptor subunit NR1a

cDNA encoding the NR1a receptor subunit was assembled downstream of an inducible cytomegalovirus minimal promoter. This latter construct (pCT-NR1a) and its inducibility were checked by Western blot upon transient

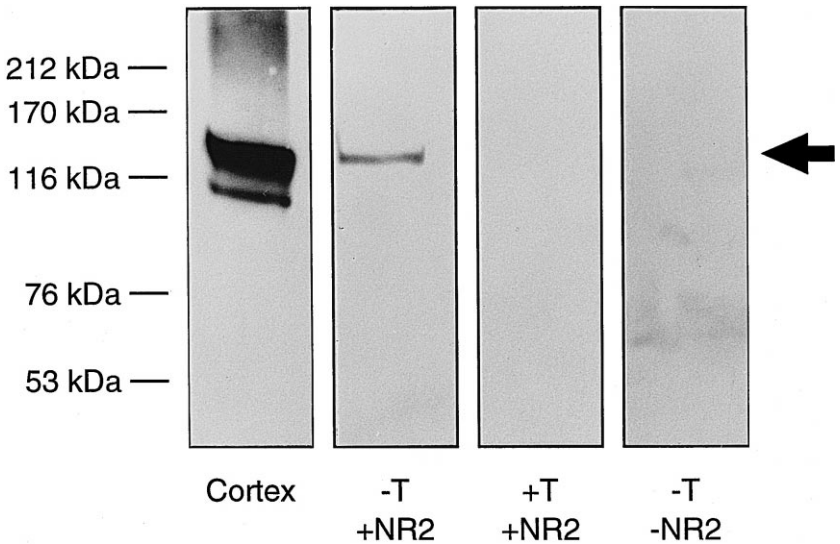

Fig. 1. Demonstration by Western blot that NR1 needs to be associated with NR2 to be expressed in stable NR1 cell lines. Western blot was performed on a NR1 expressing clone upon tetracycline removal (−T) and NR2A surtransfection (+NR2); cells being lysed 48 h after NR2 transfection. NR1 could not be seen when tetracycline was present (+T) or without NR2 supertransfection (−NR2). As a control a lane with rat brain cortex (Cortex) was added.

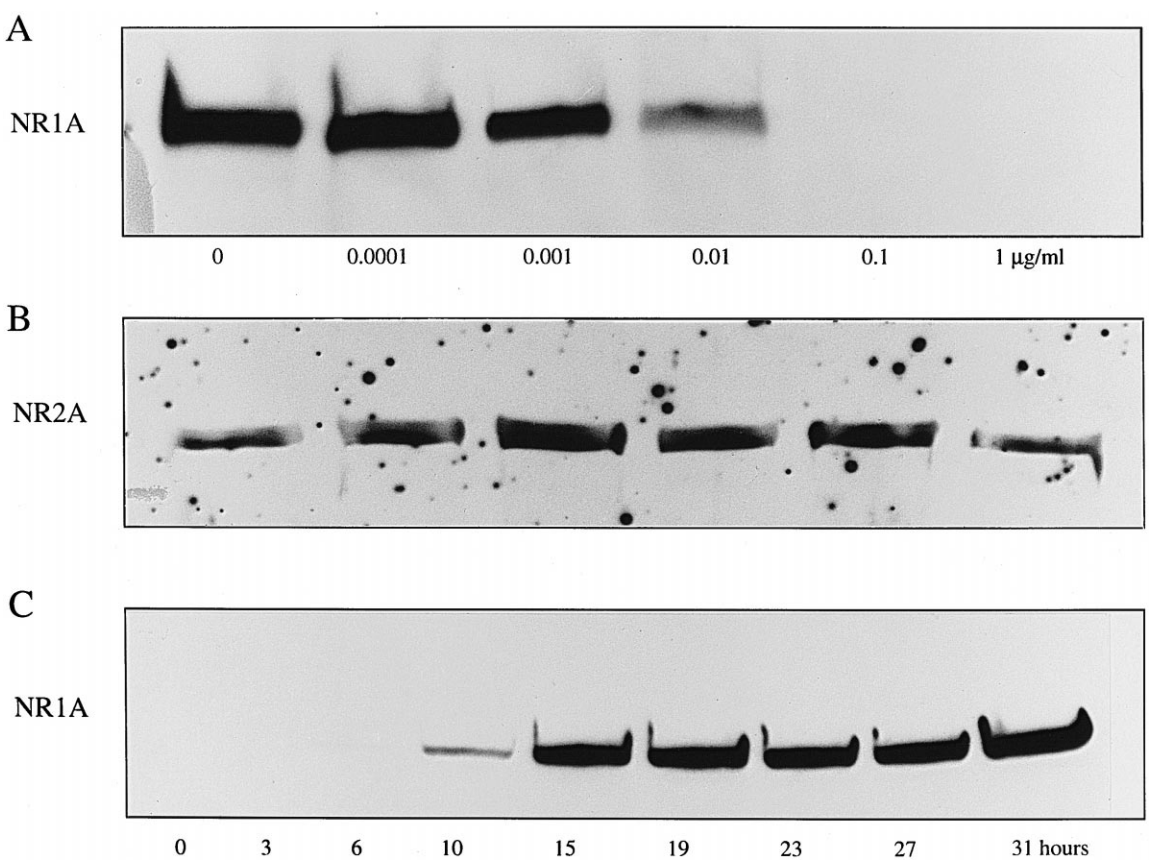

Fig. 2. Demonstration by Western blot of the effect of tetracycline on NR1 subunit expression in the binary NR1a/NR2A receptor subtype cell line AA30. Panel A: Conditional expression of the NR1a NMDA receptor subunit. Cells were incubated for 30 h with the indicated tetracycline concentrations (0 to 1 μg/ml). Cells were then lysed and Western Blots carried out with a specific NR1 antibody. Panel B: Constitutive expression of the NR2A NMDA receptor subunit. The protocol was similar to that described in A except that a NR2A/B specific antibody was used. Panel C: Time course of NR1 induction upon removal of tetracycline. Tetracycline was removed from the culture medium at the indicated times, and cells were simultaneously lysed and processed for Western blot.

transfection, and then cotransfected along with a hygromycin resistance vector. Clones isolated after hygromycin selection were screened by Western blotting. NR1a expression was only observed in the absence of tetracycline and upon transient cotransfection with NR2A (Fig. 1). Five clones which exhibited the highest level of NR1a expression as assessed by Western blotting were also assayed in binding assays using 0.4 nM [^{125}I]-MK801 subsequent to transient cotransfection with NR2A. One clone (shown in Fig. 1) exhibited the highest level of expression in both assay systems and was retained for further work. Cytotoxicity associated with this clone was also induced upon transient cotransfection with NR2A in the absence of tetracycline. Under these conditions lactate dehydrogenase (LDH) release increased by 50% compared to that observed with mock transfections or with NR2A transfections performed in the presence of tetracycline.

3.3. Establishment of a stable NR1a / NR2A cell line

NR2A cDNA was subcloned into a pcDNA3-Zeo vector and this construct then transfected into the NR1a cell line described above. Cells were maintained in the presence of tetracycline during the transfection and subsequent selection steps, and zeocin-resistant clones were isolated after 10 days of selection. In contrast, in a control experiment performed in the absence of tetracycline, no cells survived beyond 4 days of zeocin selection. This finding suggests that the cells resulting from the cotransfection were killed as a result of expression of binary functional NR1a/NR2A receptors, and even the combined presence of two NMDA receptor blockers (10 μM MK801 plus 10 μM 5,7-dichlorokynurenic acid) failed to protect the cells over one week in the absence of tetracycline. In contrast, those cells grown in the presence of tetracycline survived. The zeocin resistant clones were split into two cell culture plates and tetracycline was no longer added to one of them because clones expressing functional NMDA receptors would be expected to die upon tetracycline removal. One clone named AA30 was observed to detach from the dishes and die 24 h upon tetracycline removal. This phenomenon did not occur when cells were protected with 0.5 mM Ketamine. The presence of NR1a and NR2A subunit expression in this cell line was confirmed by Western blot using the appropriate subunit antibodies on an extract obtained from these ketamine-treated cells. Clone AA30 exhibited the strongest NR1 and NR2 signal in Western analysis. Tetracycline concentration dependence (Fig. 2A) indicated that NR1 subunit transcription can be accurately monitored by this drug with expression turned off at a concentration of 10 ng/ml. NR2 expression was constitutive, although a

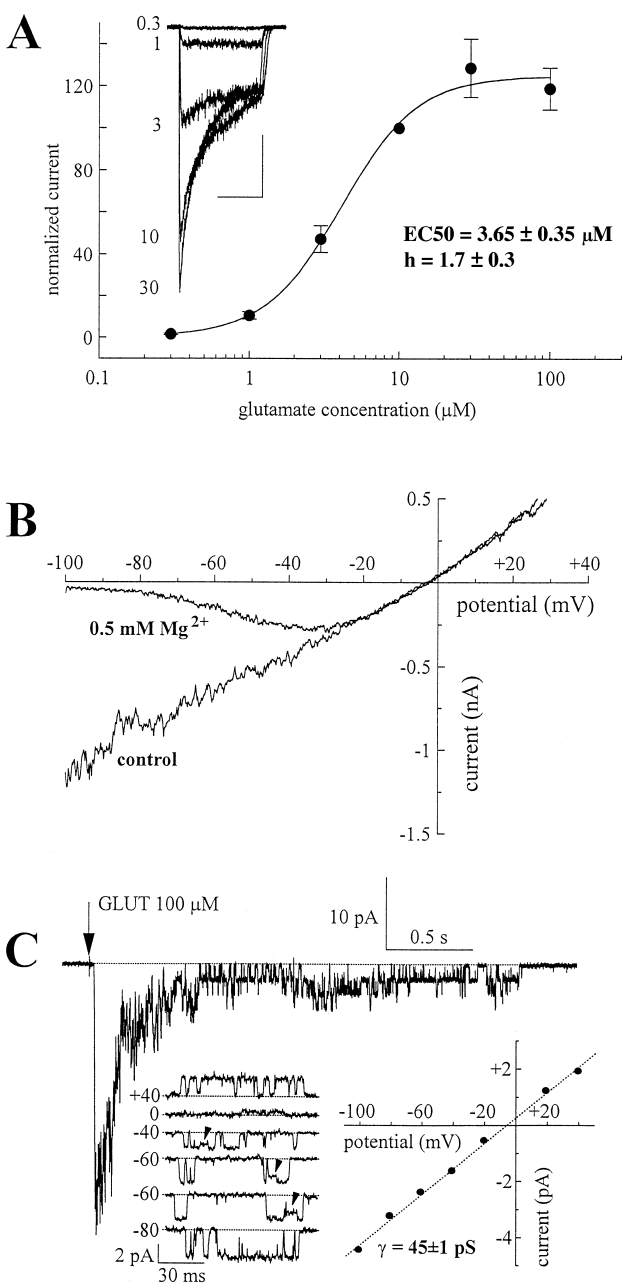

Fig. 3. Patch-clamp recordings of HEK293 cells stably expressing NR1a/NR2A receptor subunits. Panel A: Concentration-dependence curve deduced from whole cell currents evoked by the application of increasing concentrations of glutamate at a holding potential of −60 mV. Representative sweeps (0.3 to 30 μM) obtained on the same cell are shown above the curve. Vertical bar: 0.5 nA, horizontal bar: 5 s. Currents were normalized to the peak values measured during the perfusion of 10 μM glutamate. Panel B: Whole cell $I-V$ curves elicited by voltage ramps during the application to the bath of 100 μM NMDA, in the absence (control) and the presence of 0.5 mM external Mg^{2+}. Note the typical block induced by the divalent cation at negative potentials. Panel C: Single channel openings induced by the perfusion of 100 μM glutamate (arrow) in the outside out configuration at a holding potential of −60 mV (sampling rate 1 kHz and filter 0.5 kHz). The single channel $I-V$ plot revealed a main major single channel conductance of approximatively 45 pS (small arrows indicate the occasional appearance of a subconductance state of approximatively 36 pS) determined with amplitude histograms generated at indicated potentials (sampling rate 5 kHz and filter 2 kHz). All experiments were performed in the continuous presence of 10 μM glycine.

light increase was visible upon tetracycline removal (Fig. 2B). The onset of NR1 expression upon tetracycline removal could be detected as early as 6 h after the wash (Fig. 2C). Cells maintained stable NR1 and NR2 expression up until at least 25 passages.

3.4. Characterization of the NR1a / NR2A subunit expressing cell line

In order to assess functional expression of NMDA receptors at cellular membranes, we used the patch clamp technique with the AA30 clone described above between passages 15–25. As shown in Fig. 3A, an inward current was elicited on cells voltage clamped at −60 mV by the application of increasing concentrations of glutamate (EC_{50} value of 3.65 ± 0.35 μM). This current reversed at 0 mV and addition of magnesium induced a voltage-dependent block at negative potentials (Fig. 3B), in accordance with data on native NMDA currents (Mayer et al., 1984). Moreover, it was inhibited in a reversible manner with Ketamine. Representative recordings were also obtained on cell patches using the outside-out configuration (Fig. 3C). Applications of 100 μM glutamate induced openings of channels with a mean major slope conductance state of approximately 45 pS, a value close to that reported for native NMDA channels (Nowak et al., 1984) and recombinant NR1/NR2A channels (Stern et al., 1994). Occasionally a subconductance state of approximatively 36 pS was elicited (as indicated by arrows in Fig. 3C).

3.5. Development of an aequorin-based functional assay

To generate a rapid functional assay we chose to monitor Ca^{2+} entry with the Ca^{2+} reporter aequorin. The assay we developed was based upon transient expression of apo-aequorin in cells cultured in 96 well plates. Aequorin was reconstituted by a 2 h incubation with coelenterazine in the presence of AP5 (200 μM) to block NMDA receptor activity. Cells were then rinsed with Ringer–EDTA (1 mM) buffer and incubated in a magnesium and Ca^{2+} free Ringer buffer before Ca^{2+} injection. Cells were preincubated with various glutamate concentrations (1–100 μM) in the presence of 2 μM glycine and 20 μM spermine, and under these conditions glutamate gave an EC_{50} value of 4 μM (Fig. 4B). We therefore used glutamate at a final

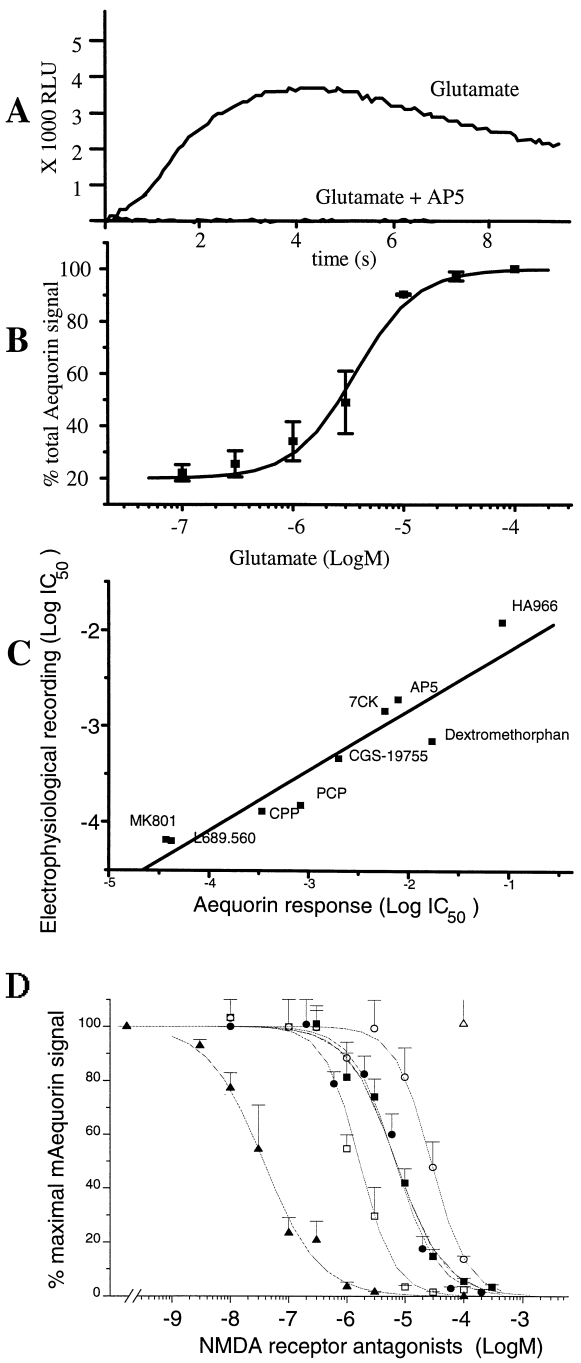

Fig. 4. Pharmacological properties of the NR1a/NR2A cell line established using an aequorin-based assay as a reporter. Panel A: Representative recordings of aequorin response to NMDA receptor activation. After a preincubation of cells with or without 200 μM AP5, luminescence was measured (one reading every 10 ms in R.L.U. relative light units) upon addition of glutamate (20 μM) in the presence of glycine (2 μM) and spermine (20 μM). Integration of these signals was carried out, with 100% and 0% values representing those obtained in the absence and presence of antagonist, respectively. Panel B: Concentration dependence of glutamate on receptor activation in the presence of glycine and spermine. Luminescence is reported as percentage of total integral. Data points are the mean ± S.D. of two independent experiments. Basal aequorin activity represented that obtained in the absence of extraneous glutamate as compared to signal in the presence of 200 μM AP5, and accounted for 20% of the maximal luminescent signal produced by 20 μM glutamate. Panel C: Correlation analysis of the IC_{50} values of selected compounds obtained in the aequorin cell-based assay and in electrophysiological recordings of the NR1a/NR2A receptor. Data were taken from Table 1 and compared using the Spearman's rho correlation method giving a coefficient of 0.945. Panel D: Representative experiment realised for some NMDA receptor antagonists or blockers assayed as described in Section 2. Each point is the mean of three luminescence measurement on independant wells. The data shown in Table 1 are the mean of three independent experiments.
Panel D: ▲ MK801 □ 5,7 dichlorokynurenic acid ● AP5 ■ Ketamine ○ Dextromethorphan △ Eliprodil.

concentration of 20 μM to induce maximum NMDA receptor activation in our subsequent assays. The signal to noise ratio attained (approximately 300:1) providing a convenient assay system to monitor Ca^{2+} entry very precisely (Fig. 4A). The concentration response curves obtained in the presence of 20 μM glutamate with different receptor antagonists were sigmoidal and reproducible (Fig. 4D).

Using this in vitro cell-based aequorin assay to measure the degree of functional activation of the NMDA receptor, we examined the pharmacological profile (Table 1) of different classes of compounds that interact with this receptor class. The rank order potencies of the IC_{50} values of AP5, CGS19755, D-CPP, 7-chlorokynurenic acid, dextromethorphan, eliprodil, HA-966, ifenprodil, L689.560, MK-801 and phencyclidine in these cell-based assays exhibited a good correlation ($r^2 = 0.945$; Fig. 4C) to those obtained in electrophysiological patch-clamp studies on NR1a/NR2A receptors (Priestley et al., 1995; Avenet et al., 1997). In addition, the IC_{50} values obtained in these cell-based assays for ketamine, phencyclidine, MK801, and dextrorphan were in the same range of potency as those previously reported in [^{125}I]I-MK801 binding studies with NR1a–NR2A transfected cells (Priestley et al., 1995; Besnard et al., unpublished results). As a control fluoxetine, a serotonin reuptake inhibitor and verapamil, a Ca^{2+} channel blocker, had no significant effect in the cell-based aequorin assay.

Table 1
Inhibition by various compounds of the glutamate-induced aequorin response in the NR1a/NR2A cell line

Compounds	IC_{50} (μM)	
	Aequorin-based assay	Patch-clamp recordings
Glutamate receptor antagonists		
AP5	7.9 ± 1.0	1.9[a]
CGS-19755	2.0 ± 0.4	0.46[a]
Glycine site antagonists		
7-Chlorokynurenic acid	5.8 ± 1.5	1.44[b], 0.63[a]
5,7-Dichlorokynurenic acid	1.5 ± 0.4	
DNQX	4.0 ± 0.8	
HA966	87 ± 5	12[a]
L689.560	0.042 ± 0.005	0.064[b], 0.041[a]
Channel blockers		
CPP	0.3 ± 0.1	0.13[a]
Dextromethorphan	17 ± 5	0.698[b]
Dextrorphan	4.5 ± 1.0	
Ketamine	12 ± 2	
MK801	0.037 ± 0.004	0.066[b]
Phencyclidine	0.83 ± 0.37	0.150[b]
Polyamine binding site		
Eliprodil	> 100	> 100[b]
Ifenprodil	> 100	> 100[b]
Compounds non related to NMDA receptor		
Fluoxetine	53 ± 11	
Verapamil	> 100	

Glutamate-induced aequorin responses in cells stably expressing the NR1a/NR2A combination were measured in the presence and absence of various concentrations of test compounds as described in Section 2 and Fig. 4.
The IC_{50} values of these compounds were calculated and represent the means ± S.E.M. of three independent experiments.
Data reported for a number of these compounds on the same subtype in either patch clamp recordings [a] with HEK293 cells (Priestley et al., 1995) or [b] in voltage-clamp recordings with *Xenopus* oocytes (Avenet et al., 1997) are given for comparison.

4. Discussion

The availability of cell lines that stably express recombinant receptors has provided convenient in vitro cellular models to characterize receptor subtypes in depth (e.g., $GABA_A$ receptor subtypes; Besnard et al., 1997). As such, given the paucity of subtype specific ligands for the NMDA receptor subfamily, cell lines that stably express NMDA receptors of predetermined composition could prove to be extremely useful in identifying selective agonists and antagonists of this receptor class and play a pivotal role in elucidating the composition and role of native subtypes. However, a major drawback to a classical approach for producing such cell-lines in the case of NMDA receptors is the high cellular toxicity seen even upon transient expression of certain NMDA receptor combinations (Anegawa et al., 1995). This cytoxicity is caused by the Ca^{2+} permeability of this ligand-gated ion channel in the presence of culture medium (Cik et al., 1993; Anegawa et al., 1995). In order to overcome this problem, we chose an alternative strategy for developing stable cell lines in which a key element depended upon an extremely performant inducible promoter system. We used this methodology to construct a binary NR1a/NR2A recombinant NMDA receptor in which the NR2A subunit was constitutively expressed in the cell line, whereas NR1a was placed under the control of the inducible promoter.

Among the various inducible promoters reported to date, few achieve induction levels greater than 100-fold. The tTA transactivator (Gössen and Bujard, 1992), which is comprised of a chimeric protein containing the tetracycline operon binding domain and the potent RNA polymerase activator VP16, however, offers this potential, provided that a cell line with an appropriate tTA level can be established. As a first step we were able to demonstrate using a luciferase reporter assay that we had indeed constructed and isolated such a cell line with this transactivator system. Control of the regulation of gene expression was very tight with a real on/off situation being created, thereby avoiding the general leakiness reported with other inducible systems (5–10 fold induction using dexamethasone induction; Gössen and Bujard, 1992). Our system,

moreover, did not display the difficulties experienced by Howe et al. (1995) also using HEK293 cells for expression of the kainate sensitive glutamate receptor subunit under the control of the tetracycline inducible promoter. Using this cell line, we subsequently constructed a further cell line in which we placed expression of the NR1a subunit under the tight control of tetracycline. This cell line was supertransfected with the NR2 subunit along with a zeocin resistance, in the presence or absence of tetracycline. In the presence of tetracycline, clones were obtained, whereas no cells survived in the absence of tetracycline, even with the constant presence of NMDA receptor antagonists during the selection procedure. Cytotoxicity in the absence of tetracycline was therefore used to screen the zeocin-resistant clones, and the clone with the strongest NR1 and NR2 expression as revealed by Western blot analysis was indeed the first to display signs of toxicity. In addition, the recombinant NMDA receptors expressed in this cell line, named AA30, were further examined by patch clamp analysis, and shown to display functional channels with biophysical and pharmacological properties similar to those previously described for recombinant NR1a/NR2A receptors (Moriyoshi et al., 1991, Monyer et al., 1992; Ishii et al., 1993; Priestley et al., 1995; Avenet et al., 1997). At the outset of our strategy to establish a stable NR1a/NR2A cell line, we initially envisaged to transfect each subunit element separately in order to select at each step the clone with the best potential, by analogy to the methodology that we previously adopted for $GABA_A$ receptors (Besnard et al., 1997). However, during the course of establishing the inducible NR1a cell line an interesting feature associated with the expression of this NR1a subunit was revealed. Although we demonstrated that the NR1 subunit was detected upon transient transfection of the NR1a construct alone in the tTA expressing cells, we were subsequently unable to detect any NR1 expressing clones among those resistant to antibiotic selection. Since functional NMDA receptors occur upon binary NR1/NR2 expression (Monyer et al., 1992; Ishii et al., 1993), we transiently transfected the NR2A subunit into the antibiotic-resistant clones obtained during the course of producing the inducible NR1a cell lines. Using Western blot analysis with specific NR1 antibodies we in fact detected the expression of NR1 protein in these NR2 subunit supertransfection experiments. A similar phenomenon has already been described in a study using PC12 cells in which a high level of NR1c messenger RNA was detected although only trace amounts of NR1 protein could be found (Sucher et al., 1993); in another study Boeckman and Aizenman (1994) established three NR1 stable cell lines in chinese hamster ovary cells but detect the NR1 protein in only one cell line although the messenger RNA is present in each cell line. Sucher et al. (1993) suggested that the expression of NR1 protein in PC12 cells might be impeded by a post-transcriptional mechanism or by rapid degradation due to post-translational mechanisms. Clearly, the NR2A subunit plays a pivotal role in stabilizing the NR1 subunit either by promoting its cell surface expression (Ehlers et al., 1995; McIlhinney et al., 1996) or by protecting this subunit from degradation as a result of receptor complex formation. In our model, therefore, conditional expression of NR1 upon NR2 co-expression should ensure that no artefactual homomeric NR1 assemblies are found, such as those that have been described to occur in the dexamethasone-inducible NR1a-NR2A cell line (Grimwood et al., 1996). As a corollary, HEK293 cells should be expected with our methodology to process combinations of other NR2 receptor subunits in appropriate binary NR1/NR2 stoechiometry.

As discussed above, the objective of establishing an inducible cell line that could stably express the NR1a/NR2A subtype was to provide an initial model system in which pharmacological analysis of NMDA receptors could be undertaken on a larger scale. As such, in addition to constructing and isolating an appropriate clone we also developed an appropriate assay system to conveniently evaluate the functional interaction of compounds with this receptor subtype. For this purpose, aequorin was developed as a potential reporter of functional activation of this receptor.

The assay that we developed was performed in 96 well plates and was based upon transient expression of apoaequorin in the NR1a-NR2A cell line. This aequorin reporter cell assay required only a small number of cells (3000 cells) but, nonetheless, generated a high signal to background ratio. This high sensitivity is explained in part by the use in our system of a novel recombinant aequorin, mAequorin, which is membrane targeted (Marsault et al., 1997). The validity of our aequorin-based reporter system was evaluated by comparing the results obtained for a variety of reference compounds to those previously reported in other studies of the NR1a/NR2A receptor. Glutamate activated this reporter system with an EC_{50} value similar to that obtained in patch clamp studies of this NMDA receptor subtype stably expressed in mammalian cells (Priestley et al., 1995). We included with glutamate in the assay the co-agonist, glycine, (Johnson and Ascher, 1987) as well as spermine, which has been suggested to reduce the pH dependence of NMDA channel activity (Traynelis et al., 1995). Also, the IC_{50} values produced in our aequorin reporter assays by the reference NMDA receptor antagonists, AP5, CGS-19755, D-CPP, L689.560, phencyclidine, MK801 and dextrorphan, gave the same rank order potencies as those obtained in patch clamp or in [^{125}I]I-MK801 binding studies with this recombinant receptor subtype (Lynch et al., 1995; Priestley et al., 1995; Grimwood et al., 1996; Avenet et al., 1997). In addition, the glycine site antagonists 5,7-dichlorokynurenic acid and 7-chlorokynurenic acid, fully inhibited Ca^{2+} influx in this aequorin assay, demonstrating the presence of a glycine co-agonist site on this recombinant receptor subtype, as previously described with native receptors (Johnson and

Ascher, 1987). Using this stable NMDA receptor cell line, eliprodil was confirmed, as previously reported (Williams, 1995; Priestley et al., 1995; Avenet et al., 1997), to have a very low affinity for the recombinant NR1a/NR2A receptor subtype. Thus, the 96-well plate format assays that we developed using aequorin proved to be an excellent reporter of the functional activation of this NMDA receptor subtype, allowing the direct comparison of potencies of NMDA receptor antagonists acting at different sites (glutamate receptor antagonists, glycine site antagonists, channel blockers). Interestingly, the IC_{50} values of certain key NMDA receptor antagonists (ketamine, MK801, phencyclidine, ifenprodil and eliprodil) in our aequorin based assay system also produced values similar to those reported in [^{3}H]MK801 and high affinity [^{3}H]dextrorphan inhibition binding assays to rat cerebral cortex membranes (Wong et al., 1988), suggesting the possible predominance of this subtype in this tissue.

The development of the novel inducible NR1a/NR2A cell line described in this study coupled with a convenient aequorin-based Ca^{2+} reporter system now provides a very powerful functional assay to identify selective ligands for this particular subtype. Moreover, the methodology set out in this report can be extrapolated to other NMDA receptor subunit combinations with the perspective of identifying novel ligands for other subtypes, or to other channels or receptors allowing a cytoplasmic Ca^{2+} rise such as G-protein coupled receptors, Ca^{2+} channels, and ligand-gated ion channels.

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