

# Chronic multichannel recordings from organotypic hippocampal slice cultures: protection from excitotoxic effects of NMDA by non-competitive NMDA antagonists

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## Abstract

In vitro neuronal damage has traditionally been evaluated by biochemical or anatomical but not by electrophysiological techniques. In the present study, we combined two newly developed technologies, an  $8 \times 8$  multi-electrode array (MED-64) and cultured hippocampal slices, to demonstrate the potential use of electrophysiological measures as index of neuronal damage. We first demonstrated the stability of electrophysiological recordings over prolonged periods of time (up to 14 days) in field CA1 of cultured hippocampal slices following electrical stimulation of the Schaffer collateral pathway. We then assessed the neurotoxic properties of NMDA and AMPA and determined that the time-course, potency, and efficacy of these two neurotoxins were similar to those assessed by other experimental approaches. We also compared the efficacy and potency of two non-competitive NMDA receptor antagonists to protect against NMDA-mediated neurotoxicity. Again, the results matched well with the results obtained from traditional techniques. Thus, this new technology might provide a new and powerful method to study the chronic effects of drugs or other experimental manipulations in an in vitro preparation. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Multichannel recording; Organotypic culture; Hippocampus; Excitotoxicity; NMDA; AMPA; MK801; Memantine

## 1. Introduction

In vitro studies of the mechanisms of neuronal cell death, and in particular of excitotoxicity, have traditionally been performed in primary neuronal cultures or in organotypic cultures of hippocampal slices (Vornov and Coyle, 1991; Choi, 1992; Bruce et al., 1995). These preparations have a number of advantages as neuronal cell death can be assessed by using a number of fluorescent markers that are related to various aspects of neuronal integrity. These methods are, in general, quite easy to implement and are amenable to large scale screening of potentially neuroprotective compounds. However, they do not provide a detailed time-course of the events leading to cell death. In addition, they do not easily discriminate the fate of different populations

of neurons (or of glial cells). Although electrophysiological techniques would seem better suited to provide a continuous record of the events leading from the initial insults to the cell demise, they have not been typically used to study neuronal degeneration. This is largely due to the limitations of traditional electrophysiological recordings that sample one or a few neurons. Furthermore, traditional recording techniques are limited to a short period of time in in vitro preparations that at most lasts 6–10 h. Chronic recording in in vitro preparations has been difficult to implement using such techniques.

Two recent technological advances have considerably eliminated these limitations. First, several multi-electrode arrays have been developed to simultaneously record the activity of large ensemble of neurons in neuronal cultures (Pine, 1980; Gross et al., 1982), organotypic cultures (Stoppini et al., 1997; Egert et al., 1998), and acute slices (Novak and Wheeler, 1988; Oka et al., 1999; Gholmieh et al., 2001). Commercially available devices now allow to record from as many as

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64 electrodes uniformly distributed in a brain slice ([www.med64.com](http://www.med64.com); [www.multichannelsystems.com](http://www.multichannelsystems.com)). In a typical hippocampal slice, these arrays can monitor the electrophysiological status of neurons in all hippocampal subfields (Stoppini et al., 1997; Egert et al., 1998; Oka et al., 1999). The second advance was accomplished by Yamamoto et al. (1989), Stoppini et al. (1991), who devised a new and simple culture system for organotypic slices that replaced the roller-tube technique developed by Gahwiler (1981). Using this technique, several laboratories have demonstrated that these cultured slices (generally prepared from postnatal day 10–12 old rats) exhibit surprisingly adult-like properties at the morphological, biochemical and electrophysiological levels (Buchs et al., 1993; Muller et al., 1993; Bahr et al., 1995; Vanderklisch et al., 1995). The present study was directed at combining these two techniques to show that it is now possible to study in great details the progression of neuronal damage elicited by prolonged treatment with excitatory amino acids. Thus, we report here a detailed time-course of the changes in electrophysiological responses recorded over several days during continuous application of AMPA or NMDA in cultured hippocampal slices. We also utilized this approach to evaluate the protection provided by non-competitive antagonists of NMDA receptors.

## 2. Materials and methods

### 2.1. Hippocampal slice cultured on the MED probes

Before use, the MED probes (Panasonic; MED-P530AP, each electrode:  $50 \times 50 \mu\text{m}$ , interpolar distance:  $300 \mu\text{m}$ ) were soaked in 70% ethanol for 15 min, dried up in a clean bench and sterilized with UV radiation for 15 min. The surface of the probes was treated with 0.1% polyethylenimine and 25 mM borate buffer, pH 8.4, overnight at room temperature. The probe surface was dried and rinsed three times with sterile distilled water. Finally, the probes were filled with culture medium and stored in a  $\text{CO}_2$  incubator until use (for at least 1 h). The culture medium was a 2:1 mixture of Basal Medium Eagle (Sigma; B9638) and Earle Balanced Salts Solution (Sigma; E7510), supplemented with the following compounds (in mM): NaCl (20),  $\text{NaHCO}_3$  (5),  $\text{CaCl}_2$  (0.2),  $\text{MgSO}_4$  (1.7), glucose (48), HEPES (26.7), 5% horse serum (GIBCO; 26050) and 10 ml/l penicillin–streptomycin (GIBCO; 10378) were added and the pH was adjusted to 7.2.

All procedures for culture preparation were carried out under a sterilized bench. Eleven-day-old Sprague–Dawley rats were sterilized with 70% ethanol, sacrificed by decapitation following anesthesia and the whole brain was removed. The brains were immediately soaked in sterile ice-cold MEM (pH 7.2; GIBCO; 61100),

supplemented with HEPES (25 mM), Tris–base (10 mM), glucose (10 mM) and  $\text{MgCl}_2$  (3 mM). Appropriate portions of the brain were trimmed by hand and the remaining brain block was placed on the ice-cold stage of a vibrating tissue slicer (Leica; VT1000S). The thickness of the slices was set at  $200 \mu\text{m}$ . The slices were gently taken off from the blade with a pipette. Each slice was trimmed, placed on the center of the MED probe, which was previously coated as mentioned above, and positioned to cover the  $8 \times 8$  microelectrode array. After positioning the section on the MED probe, the cutting solution was removed and the culture medium was added to the slice up to an interface level (approximately  $250 \mu\text{l}$ ). Sterile distilled water was added around the probe to increase humidity and prevent over-drying of the culture medium in the MED probe. The slices on the MED probes were stored in a  $\text{CO}_2$  incubator at  $34^\circ\text{C}$ . The medium was exchanged with half volume every day. Slices were cultured for about 10 days prior to initiating electrophysiological recordings.

### 2.2. Electrophysiological recordings

For electrophysiological recordings, the MED probes containing the slices were removed from the incubator and placed in a smaller  $\text{CO}_2$  incubator at  $34^\circ\text{C}$  and connected to the stimulation/recording component of MED-64. The medium was replaced with sterile artificial cerebro-spinal fluid (ACSF) of the following composition (in mM): NaCl (124),  $\text{NaHCO}_3$  (26), glucose (10), KCl (3),  $\text{NaH}_2\text{PO}_4$  (1.25),  $\text{CaCl}_2$  (2),  $\text{MgSO}_4$  (1), and HEPES (10). Evoked field potentials at all 64 sites were recorded simultaneously with the multichannel recording system (Panasonic; MED64 system) at a 20 kHz sampling rate. One of the planar microelectrodes of the 64 available was used for cathode of stimulation. Bipolar constant current pulses ( $10\text{--}45 \mu\text{A}$ , 0.1 ms) were produced. To collect typical responses in field CA1, one of the electrodes in the Schaffer collateral fibers was selected as a stimulating electrode while another one in stratum radiatum was selected as a recording electrode. Synaptic responses were recorded at eight-step stimulation intensities ( $10\text{--}45 \mu\text{A}$ , 5  $\mu\text{A}$  steps). This recording was first carried out in the absence of any drug to establish a baseline. After each recording session, ACSF was replaced with culture medium and the slices in the probes were returned to the  $\text{CO}_2$  incubator. For drug treatment, culture medium containing appropriate concentrations of various drugs was applied. The recording procedure was repeated after 3 h, 1 day, 2 days, 3 days and afterwards every other day in the presence or absence of drug treatment. All drugs were purchased from Sigma.

### 2.3. Data analysis

One recording channel for analysis was selected among the electrodes located in stratum radiatum of field CA1. Maximum amplitudes of field EPSPs were measured. These measurements were carried out at each stimulus step, and data were plotted as input–output (I/O) curves. All data are presented as mean  $\pm$  S.E.M. One-way analysis of variance (ANOVA) was employed for statistical analysis.

### 2.4. Propidium iodide (PI) uptake measurement

We used a standard PI uptake method (Bruce et al., 1995; Noraberg et al., 1999) to compare electrophysiological data and more traditional measures of neurodegeneration. After 10 days in vitro (DIV), 4.6  $\mu$ g/ml PI was added to the culture medium. This concentration was used in the medium at all subsequent medium changes. Three hours after PI application, fluorescence images were captured by fluorescence microscopy (Zeiss; Axiovert 200M, 10 $\times$ ) using a standard rhodamine filter and digital CCD camera (Zeiss; AxioCam). After capturing baseline fluorescence, the cultures were exposed to appropriate concentrations of either NMDA or AMPA. The PI fluorescence in the individual cultures was recorded again after 2 days. At the end of experiments, all neurons were killed by exposure to 50 mM glutamate for 1 h followed by incubation for 24 h in normal culture medium (with PI) to measure the fluorescence intensity corresponding to the maximal fluorescence in individual cultures. The pixel intensity was measured for field CA1 with Image J 1.27z (NIH). The percent neuronal death obtained after 2 days of treatment with excitotoxins was calculated as percent of final fluorescence ( $F_{\text{fin}}$ ) minus baseline fluorescence ( $F_0$ ) according to the formula: % neuronal death =  $(F - F_0) / (F_{\text{fin}} - F_0) \times 100$ , where  $F$  is the PI fluorescence measured after 2 days of treatment.

## 3. Results

### 3.1. Stability of long-term recordings

In order to address the question of how neurotoxic agents affect synaptic responses in hippocampal pathways, stable long-term monitoring of synaptic transmission is required. We cultured hippocampal slices directly on the surface of the MED probe, which allowed us to conduct long-term extracellular recordings in CA1 and other hippocampal subfields. Directly culturing hippocampal slices on polyethylenimine-coated probes resulted in a tight adhesion of the slices on the probes, and provided for stable maintenance of the stimulating and recording sites. Typically, one pair of electrodes

(one stimulating and one recording electrode) was selected in CA1 to stimulate Schaffer collateral afferents and to extracellularly record evoked postsynaptic field potentials (fEPSPs) with the best possible quality.

In agreement with prior studies (Stoppini et al., 1991), slices prepared from postnatal day 10–12 rats provided optimal results when tested electrophysiologically after culturing on the MED probe in static conditions with interface levels of culture medium. Example of a slice cultured on the MED probe for 1 week in vitro as well as positions of the electrodes are depicted in Fig. 1A. Under our experimental conditions, no obvious morphological changes were noticed during at least 2 weeks in culture on the MED probes. Slight to moderate migration of the cells out of the slice was observed, normally starting after 14–20 days. Some flattening of the slices also occurred after 2 weeks in vitro, which did not appear to interfere with fEPSP recordings. During the first 7 days of in vitro culturing, we observed some increase in the amplitude of fEPSPs; however after 7–10 days, the responses stabilized and remained stable over subsequent recording periods. These results were consistent with the findings of Muller et al. (1993). As a result, all experiments reported here were done after at least 10 days of culturing slices on the MED probes.

fEPSPs were recorded at 20 s interval using a paired-pulse stimulation paradigm with an inter-pulse interval of 50 ms. Paired pulse facilitation was typically observed (Fig. 1B). Slope and decay time of the responses were remarkably similar to those recorded in acutely prepared slices using the same system (Fig. 2C). However, a delay after the stimulus artifact was generally observed in the culture conditions, possibly due to the lack of myelination of axons in cultured slices as previously reported (Buchs et al., 1993).

In order to obtain a better evaluation of the effects produced by tested substances, control experiments were carried out in parallel with those including test substances. In all experiments, the amplitude of fEPSPs in control slices was used as an index of the preparation's 'health' during the recording period. Various stimulation current intensities (ranging from 10 to 45  $\mu$ A) were used to obtain corresponding fEPSPs (Fig. 2A). Amplitudes measured at the peaks of fEPSPs were plotted as a function of the stimulation intensity thus providing I/O curves (Fig. 2B), which were also very similar to those obtained in acute hippocampal slices (Fig. 2D).

In the chronic drug application protocol, stability of the recordings was tested by comparing fEPSP amplitudes measured at the beginning of the experiments with those measured after 3, 24, 48, and 72 h at the same stimulation intensity (Fig. 1C). For data analysis, the amplitude of the maximal (plateau) value of the I/O curve was used to determine stability of the recordings. Typically, it corresponded to responses obtained by stimulation intensities of 30–40  $\mu$ A (Fig. 2). Recordings

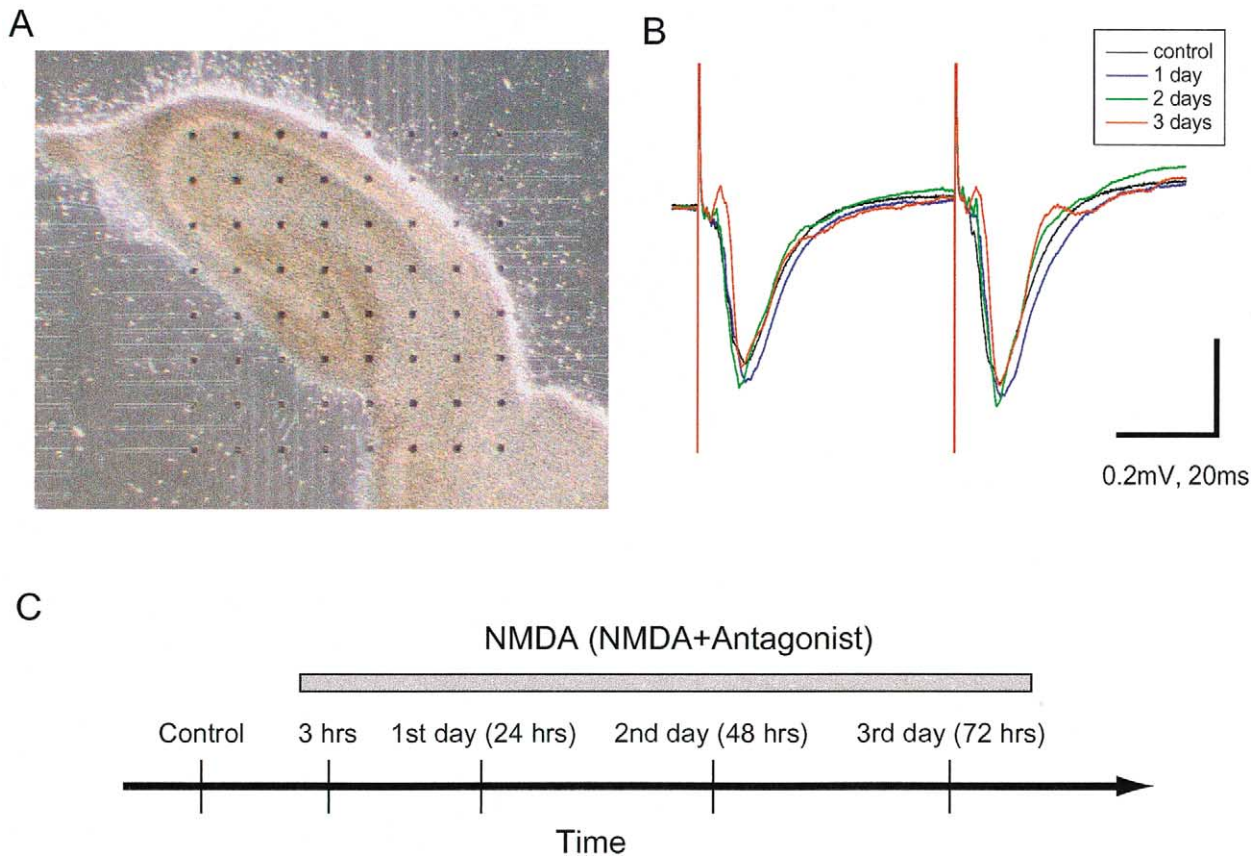


Fig. 1. Experimental design used to perform chronic recording in cultured hippocampal slices. Hippocampal slices from 11-day-old rats were maintained on the MED-64 probes for extended periods of time (A). Paired-pulse stimulations were delivered to various electrodes and extracellular field potentials were recorded (B). Standard protocols to evaluate the effects of various agents on evoked responses consisted in obtaining a baseline response, then applying the drugs for various periods of time and evaluating synaptic responses again (C). Baseline responses were measured from 10 DIV slices on the MED probes. The corresponding age of the slices at 3, 24, 48, and 72 h after drug application was 10, 11, 12, and 13 DIV, respectively.

in 23 control experiments indicated that the relative amplitude of fEPSP after 3 days of chronic recording was  $107 \pm 4\%$  ( $n = 23$ ) of the control value, indicating a slight increase in the amplitude over the period of measurements (10–13 DIV).

For longer incubation periods (i.e. 20 DIV), the response amplitude was also  $107 \pm 4\%$  of control values ( $n = 2$ ). In one slice, recordings were even possible after 45 DIV, although with approximately a 50% reduction in amplitude. However, in the present study, all results are presented for slices cultured no longer than 20 DIV.

### 3.2. NMDA and AMPA toxicity

Numerous studies have used selective glutamate receptor agonists for producing pathological conditions in the brain in order to study mechanisms of neuronal death and neurotoxicity (see Lee et al., 1999, for a review). In our case, NMDA and AMPA were chosen as excitotoxic agents to compare electrophysiological parameters with traditional parameters of neurodegeneration, and to gain additional information regarding the

mechanisms contributing to the loss of synaptic function in excitotoxic hippocampal injury. Long-term incubation of cultured hippocampal slices in the presence of either NMDA or AMPA resulted in dose-dependent decreases in synaptic responses. Representative I/O relationships and fEPSP recordings before and at various times after incubation in the presence of NMDA (10  $\mu\text{M}$ ) or AMPA (1  $\mu\text{M}$ ) are shown in Fig. 3. After 3 h of incubation in the presence of 10  $\mu\text{M}$  NMDA, the maximal amplitude of synaptic responses was reduced to  $18 \pm 4\%$  ( $n = 3$ ) of control values and this decrease did not change significantly during subsequent incubation periods (Fig. 3A, representative recordings are shown in Fig. 3C). When 1  $\mu\text{M}$  AMPA was applied for 3 h, the decrease in synaptic response was smaller, and the responses continued to decrease at subsequent time points, and stabilized after 1 day (Fig. 3B, representative recordings are shown in Fig. 3D).

The mechanisms underlying the reduction in amplitude of synaptic responses produced by incubation with these two excitotoxins were further studied by applying various concentrations of NMDA and AMPA for 2

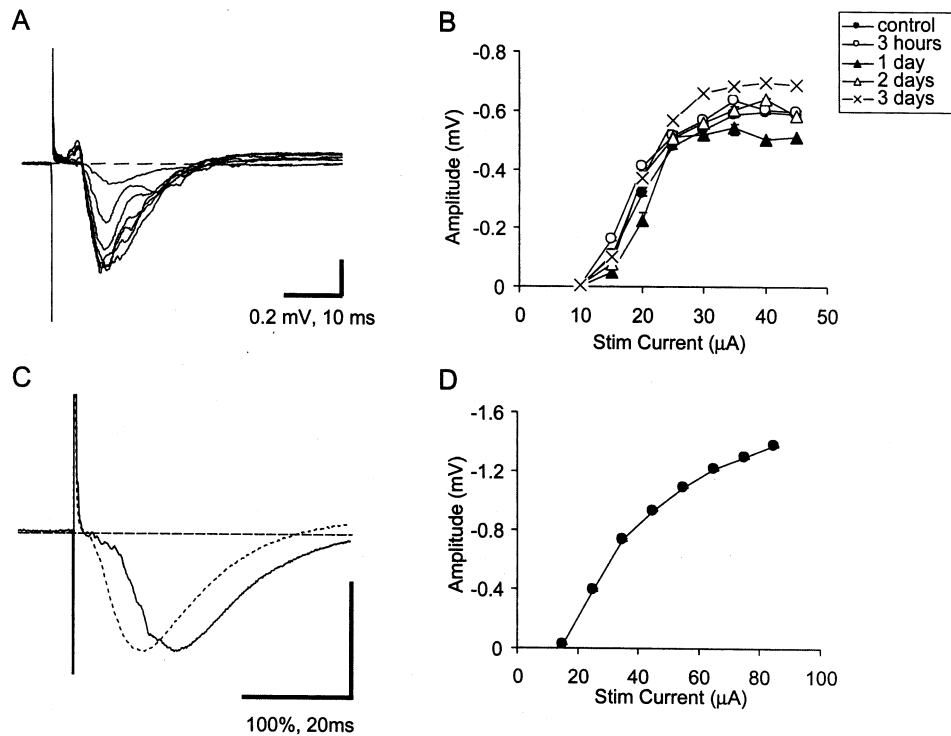


Fig. 2. I/O relationships at various time points under control conditions. Stimulations were delivered at various intensities to the Schaffer collateral pathway and corresponding responses were recorded in stratum radiatum of CA1 (A). I/O relationships were calculated for the first responses after various times in culture (B). Comparison of responses recorded in cultured (solid lines) and acute hippocampal slices (dashed line), which were prepared from 21-day-old rats. For comparison, each response was normalized to its maximum amplitude (C). I/O relationships were calculated in acute slices (D).

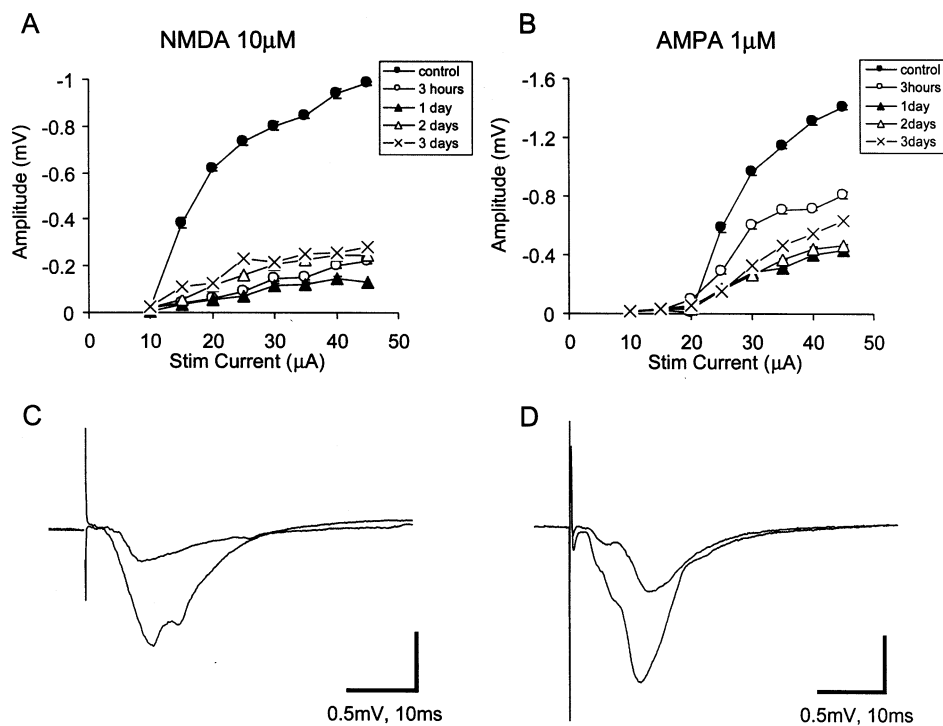


Fig. 3. Chronic effects of NMDA and AMPA on synaptic responses in CA1. After collecting baseline responses, slices were treated with NMDA (10  $\mu\text{M}$ ; A, C) or AMPA (1  $\mu\text{M}$ ; B, D) for the indicated periods of time, and I/O relationships were obtained. The amplitudes of synaptic responses were plotted as a function of the intensities of stimulation (typical cases are shown) (A, B). Representative traces of responses obtained at maximal stimulation intensity before and 3 days after excitotoxin application (C, D, average of three sweeps).

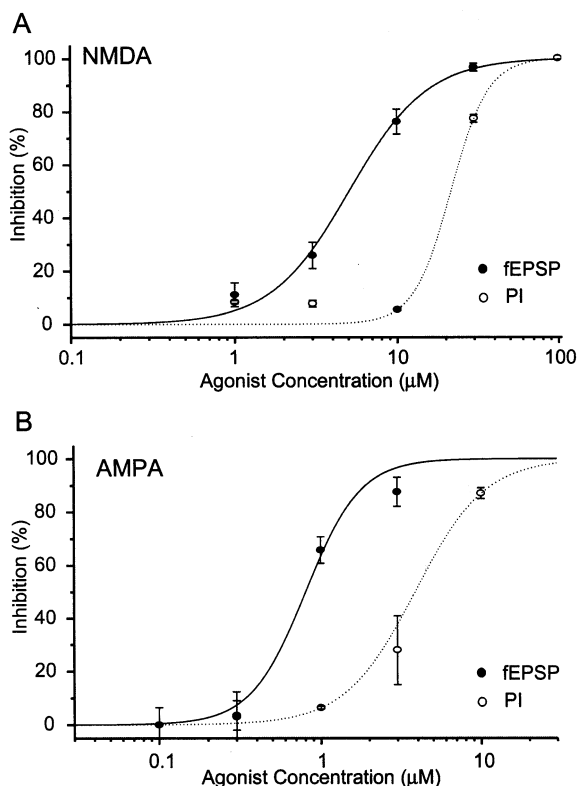


Fig. 4. Chronic effects of various concentrations of NMDA and AMPA on synaptic responses and neuronal damage in CA1. Synaptic responses to maximum stimulation intensities were recorded 48 h after chronic treatment of cultured hippocampal slices with NMDA (A) or AMPA (B). Results were calculated as percent inhibition of baseline values and represent means  $\pm$  S.E.M. of three experiments (solid circles). Neuronal damage (open circles) was assayed with the PI uptake method as described in Section 2. Results were calculated as percent maximal damage and represent means  $\pm$  S.E.M. of two experiments. Solid and dashed lines represent the best-fit curves for synaptic responses and PI uptake.

days. As in the control experiments, the plateau values of the respective I/O curves were used for the analysis (Fig. 4). Fitting the dose–response curves with the Hill function generated EC<sub>50</sub> values of  $5.2 \pm 0.5 \mu\text{M}$  for NMDA and  $0.81 \pm 0.1 \mu\text{M}$  for AMPA, with Hill coefficients of  $1.7 \pm 0.2$  and  $2.6 \pm 0.8$ , respectively. For comparison, we plotted in the same figure dose–response curves obtained with the PI uptake method (Fig. 4, open circles and dashed lines). EC<sub>50</sub> values of PI uptake method were  $21.6 \pm 3.4 \mu\text{M}$  for NMDA and  $3.87 \pm 0.1 \mu\text{M}$  for AMPA and Hill coefficients of  $3.7 \pm 1.0$  and  $2.0 \pm 0.1$ , respectively. The dose–response curves obtained with electrophysiology were shifted toward the left and were also less steep (at least for NMDA) than those observed with the PI method, suggesting that these two methods reveal different cellular mechanisms activated by NMDA and AMPA.

To further address this question, we investigated the time-course for recovery of synaptic responses after agonist removal. At the end of incubation in the

presence of  $10 \mu\text{M}$  NMDA for 40 min, 3 h, 1 day, and 3 days, the amplitudes of synaptic responses were  $48 \pm 12$ ,  $22 \pm 7$ ,  $13 \pm 3$ , and  $11 \pm 3\%$  of control values, respectively ( $n = 3$ ). However, following 1 h of NMDA wash-out under the same conditions, synaptic responses were  $109 \pm 4$ ,  $66 \pm 5$ ,  $52 \pm 2$ , and  $18 \pm 3\%$  of the initial amplitude, respectively (Fig. 5), indicating that synaptic responses in fact gradually decrease and become irreversibly diminished only after 3 days of continuous treatment with NMDA. Similar results were obtained for AMPA: a 40 min incubation in the presence of  $3 \mu\text{M}$  AMPA led to a large decrease in the amplitude of synaptic responses ( $12 \pm 5\%$  of control values) and the responses recovered to up to  $93 \pm 2\%$  of control after AMPA was removed for 1 h ( $n = 3$ ); 3 days of incubation in the presence of  $1 \mu\text{M}$  AMPA decreased synaptic response amplitudes to  $46 \pm 4\%$  ( $n = 3$ ) and no recovery was observed even after 24 h of AMPA removal.

Thus, partial recovery of synaptic responses at early time-points in our experiments suggests that it is unlikely that only neuronal degeneration contributes to the decrease in synaptic transmission. Additional suppression of synaptic transmission may occur due to agonist-induced opening of glutamate receptor channels and subsequent neuronal depolarization.

### 3.3. Comparison of protection elicited by various NMDA antagonists

To validate the ability of our system to discriminate drugs with different neuroprotective properties, the potency of two distinct non-competitive NMDA receptor antagonists was determined. MK-801 is a known high-affinity antagonist, while memantine is a lower affinity antagonist and is currently used in clinical treatments (Parsons et al., 1999). Based on the dose–

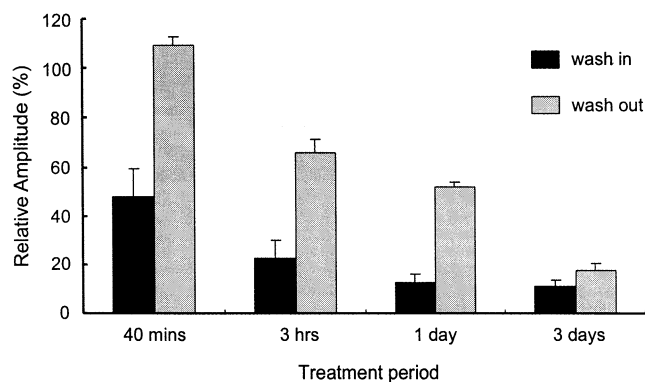


Fig. 5. Partial reversibility of NMDA-induced decrease in synaptic responses in CA1. Synaptic responses to maximum stimulation intensities were recorded at the end or 1 h after treatment with  $10 \mu\text{M}$  NMDA for the indicated periods of time. Results were expressed as percent of baseline responses and are means  $\pm$  S.E.M. of three experiments.

response analysis performed above (Fig. 4A), 10  $\mu$ M NMDA was chosen as the concentration of agonist for the antagonist tests. Since we were interested in evaluating the neuroprotective effects of these antagonists in chronic application, we did not study their effects before the first 3 h time point. After performing control measurements, slices were incubated in culture medium containing 10  $\mu$ M NMDA and 1  $\mu$ M MK-801 for various periods of time. Under these conditions, synaptic responses were only slightly depressed from 3 h up to 3 days of incubation (Fig. 6A), indicating that 1  $\mu$ M MK-801 almost completely protected synaptic transmission against NMDA-mediated neurotoxicity, a result in good agreement with previous studies using different markers of synaptic damage (Peterson et al., 1989; Pringle et al., 2000; Kristensen et al., 2001). Relative amplitude of synaptic responses measured at stimulation intensities corresponding to the plateau of I/O curves after 3 days of incubation was  $91 \pm 6\%$  of control ( $n = 3$ ). When memantine was used as an antagonist, the pattern of protection was relatively similar, as synaptic responses were significantly protected for up to 3 days of incubation in the presence of 10  $\mu$ M NMDA and

30  $\mu$ M memantine. However, the degree of protection was much smaller than that provided by MK-801, as only  $78 \pm 5\%$  ( $n = 3$ ) of the initial amplitude was preserved under these conditions (Fig. 6B). The concentration dependency of memantine protection was studied using 10  $\mu$ M NMDA and various concentrations of memantine. The dose–response curve was fitted with the Hill equation and provided values for IC<sub>50</sub> of  $6.9 \pm 1.6$   $\mu$ M, with a Hill coefficient of  $1.1 \pm 3$  (Fig. 6C).

### 3.4. Chronic effects of antagonists

Despite its relatively high affinity, MK-801 is not used clinically to protect CNS from neurodegenerative and other kinds of disorders. One of the reasons for its inadequateness for medical treatment is related to the almost irreversible nature of its binding to the NMDA receptors (Parsons et al., 1999), and its neurotoxic properties (Fix et al., 1993; Horvath et al., 1997). In our experiments, incubation of the slices in the presence of 1  $\mu$ M MK-801 alone resulted in decreased synaptic responses after 1 day. Interestingly, synaptic responses were not affected after 3 h of incubation (Fig. 7A).

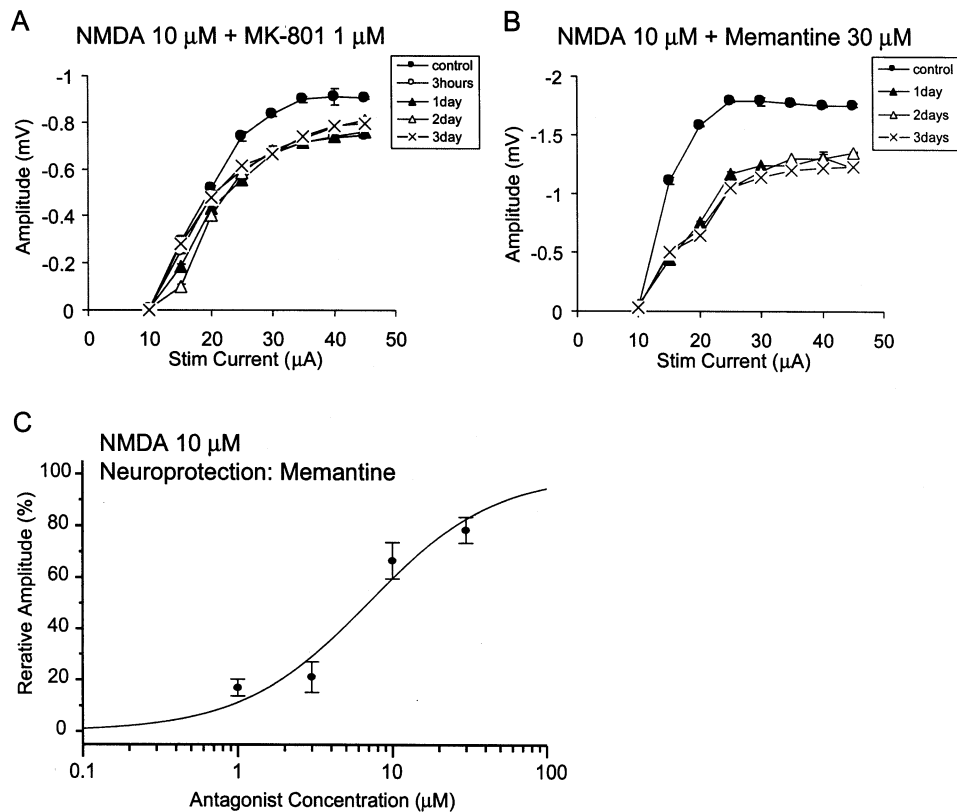


Fig. 6. Effects of MK801 and memantine on NMDA-induced decrease in synaptic responses in CA1. After collecting baseline responses, slices were treated with 10  $\mu$ M NMDA and 1  $\mu$ M MK801 (A) or 10  $\mu$ M NMDA and 30  $\mu$ M memantine (B) for the indicated periods of time. I/O relationships were obtained and the amplitudes of the synaptic responses were plotted as a function of the intensities of stimulation (typical cases are shown). Synaptic responses elicited by stimulation at maximum intensity were recorded 72 h after chronic treatment of cultured hippocampal slices with 10  $\mu$ M NMDA in the presence of various concentrations of memantine (C). Results were calculated as percent of responses recorded under control conditions (i.e. in the absence of NMDA) and represent means  $\pm$  S.E.M. of three experiments.

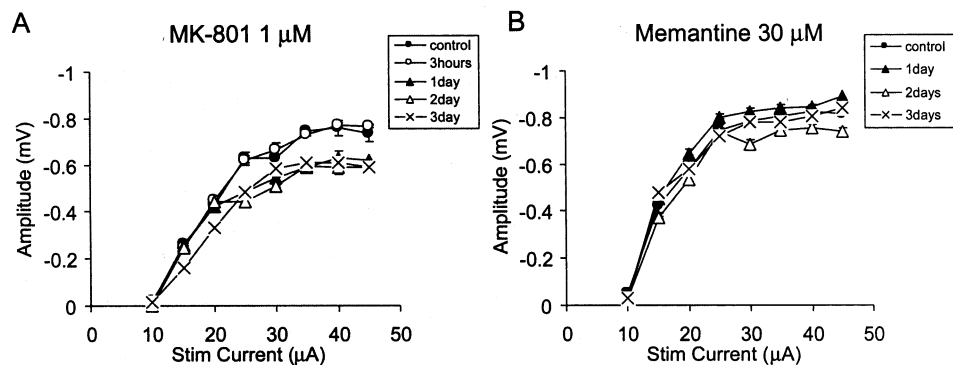


Fig. 7. Chronic effects of MK801 and memantine on synaptic responses in CA1. After collecting baseline responses, slices were treated with 1 μM MK801 (A) or 30 μM memantine (B) for the indicated periods of time. I/O relationships were obtained and the amplitudes of the synaptic responses were plotted as a function of the stimulus intensity (typical cases are shown).

However, after 1, 2, and 3 days of incubation, the amplitudes of responses were decreased to  $83 \pm 6$ ,  $79 \pm 11$ , and  $76 \pm 10\%$  ( $n = 3$ ) of control, respectively. These decreases in amplitude were not significantly different from the decrease in amplitudes produced by 1 μM MK-801 in the presence of 10 μM NMDA (Fig. 6A). In contrast to MK-801, long-term incubation in the presence of memantine even at 30 μM did not result in a significant decrease in synaptic transmission (Fig. 7B).

Results obtained with chronic application of NMDA receptor agonist or antagonists are summarized in Fig. 8. Control responses were measured in 23 slices

and were compared with the results obtained after incubation during 3 days in the presence of NMDA, NMDA plus antagonists or antagonists alone. Our results indicate that the large (70–80%) decrease in synaptic responses produced by incubation in the presence of 10 μM NMDA could be almost completely prevented ( $91 \pm 6\%$ ,  $n = 3$ ) by co-incubation with 1 μM MK-801 (not significantly different than control values). Protection was substantially weaker for memantine even at higher concentrations, as only  $78 \pm 5\%$  ( $n = 3$ ) of synaptic responses remained after treatment with NMDA and 30 μM memantine ( $P < 0.05$  as compared to control values). However, it is important to note that incubation with memantine (30 μM) but not MK-801 (1 μM) in the absence of NMDA resulted in responses that were statistically identical to those obtained under control conditions (control vs. memantine:  $P = 0.267$ , control vs. MK-801:  $P = 0.010$ ).

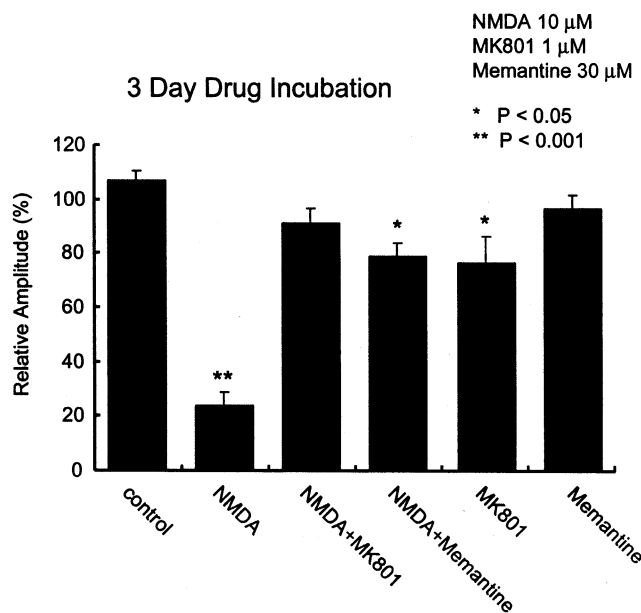


Fig. 8. Comparison of the effects of various chronic treatments on synaptic responses in CA1. Synaptic responses to stimulation at maximal intensity were recorded in cultured hippocampal slices 3 days after treatment with various drugs or combinations of drugs. Results represent amplitudes of synaptic responses; they are expressed as percent of the values recorded under control conditions and are means  $\pm$  S.E.M. (control:  $n = 23$ , other cases:  $n = 3$ , \* $P < 0.05$ , \*\* $P < 0.001$ ).

#### 4. Discussion

The present study indicates that the combination of two new technologies, i.e. the MED-64 multielectrode array, and the cultured hippocampal slices, provides a new and powerful tool to obtain long-term recording from postnatal brain tissue. In our hand, we could obtain stable recordings for at least 20 days, a period of time more than suitable to study long-term effects of numerous pharmacological or genetic manipulations. Furthermore, the characteristics of the responses (I/O responses, paired-pulse facilitation, waveforms) were remarkably similar to those obtained with more traditional electrophysiological techniques as well as with acute hippocampal slices. The delay in synaptic responses we systematically observed was very likely due to the sparse myelination of axons previously described in this preparation (Buchs et al., 1993). Previous reports of chronic recordings in cultured hippocampal slices

were generally limited to a few days (Egert et al., 1998; Dupont et al., 1999). We believe that this technical approach, which is relatively easy and inexpensive, will open the way to provide answers to a wide range of questions that were thought impossible to address with present technologies.

We illustrated the validity of this experimental approach by evaluating features of neurotoxicity of NMDA and AMPA and by comparing the neuroprotective effects of two non-competitive antagonists of NMDA receptors, MK-801 and memantine. Our data indicated a very rapid decrease in synaptic responses elicited by chronic treatment with NMDA or AMPA. Within 3 h of treatment with either agonist, synaptic responses had already decreased by 85 and 55%, respectively. By 24 h after treatment initiation, synaptic responses were decreased by about 90 and 75%, respectively. While the data obtained at 48 h match relatively well with those obtained from other techniques used to assess neuronal damage, and in particular those obtained with the PI uptake method (note that our data are almost identical to those reported by Kristensen et al., 2001), a few discrepancies are worth discussing. The rapid time-course of decreased synaptic responses does not match that reported with the PI uptake method as very little neuronal damage was observed after 3 h of incubation with even much higher concentrations of NMDA in cultured hippocampal slices (Bruce et al., 1995). The large decrease in synaptic responses observed at 3 h could thus be due to a prolonged depolarization elicited by NMDA or AMPA treatment. This seems likely as synaptic responses were able to almost completely recover following NMDA or AMPA wash-out after a 40 min treatment, and still partially recovered following a 24 h treatment. In particular, the percent decrease in the amplitude of synaptic responses measured 1 h after NMDA wash-out following a 1 day treatment with 10  $\mu$ M NMDA for 1 day is almost identical to the decrease in neuronal viability measured in CA1 with the PI uptake (Kristensen et al., 2001). Second, the dose–response curves for both NMDA and AMPA determined after 48 h of chronic treatment were shifted to the left and were less steep than those observed with the PI method (see Fig. 4). This could be due to the existence of additional effects of NMDA or AMPA and in particular to prolonged depolarization, as it is likely to precede neuronal damage, and thus to occur at lower concentrations than those neurotoxic.

The characteristics of the protective effects of non-competitive antagonists of NMDA receptors against NMDA-induced decrease in synaptic responses also matched well with the data obtained from the PI uptake method, and in particular our data indicated that memantine is much less neuroprotective than MK-801 (Pringle et al., 2000). However, our data also indicated

that memantine might not have some of the undesirable side-effects of MK-801, as no effect of memantine on synaptic responses were detected after chronic treatment with relatively high concentrations. This contrasts with the small but significant decrease in synaptic responses elicited by MK-801. Other studies have reported neurotoxic effects of MK-801 in various brain structures (Fix et al., 1993; Horvath et al., 1997). It might be interesting to compare the neurotoxic effects of MK-801 revealed with our technique with other techniques used for neurodegeneration.

In conclusion, the present studies clearly demonstrate that chronic multi-site recordings in an in vitro preparation represents a new methodology to assess the properties and mechanisms of neuronal damage. This technology should be of interest to a wide range of neuroscientists and should provide a new and powerful method to study the chronic effects of drugs or other experimental manipulations in an in vitro preparation.

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