Protective Effect of Ifenprodil against Spreading Depression in the Mouse Entorhinal Cortex

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Summary

In the brain, spreading depression (SD) is characterized by a large extracellular DC shift, a massive failure of ion homeostasis and a transient cessation of neuronal function. Clinically, SD is believed to be involved in various neurological disorders including migraine and cerebrovascular diseases. The propagation of cortical SD requires the release of glutamate, and NMDA receptors play a crucial role in this process. Here, we have isolated the NMDA-receptor-mediated component of extracellularly recorded field EPSPs (fEPSPs) in layers 2-3 of the entorhinal cortex of murine brain slices. In the absence of GABA_A and AMPA receptor mediated synaptic transmission, stimulation of layer 6 afferents every 15 - 90 s elicited spontaneous SD on average within 18.5 min after the start of the stimulation. In the presence of ifenprodil, an NR2B receptor subunit-selective NMDA receptor antagonist, the occurrence of SD was nearly abolished. Our results are consistent with an important role of NR2B subunits in triggering SD in the entorhinal cortex.
Introduction

Spreading depression (SD), first described by Leão (1944), is characterized by a large extracellular DC shift (do Carmo and Somjen 1994), a massive failure of ion homeostasis, and a transient cessation of neuronal function (Somjen 2001). SD can be observed in most gray matter areas, but the hippocampal CA1 region and the neocortex are especially prone to SD (Somjen 2001). SD has been observed in vivo in different species, including mammals (Krivanek 1976), and in vitro brain slices (Snow et al. 1983). Both in vivo (Mayevsky et al. 1996) and in vitro (Avoli et al. 1995) recordings from human cortical tissue have shown SD activity. The association of SD with various neurological disorders is well documented (Gorji 2001), but SD has been principally linked to migraine, cerebrovascular diseases, head injury and transient global amnesia. The clinical relevance of SD has been dealt with by a recent review (Gorji 2001).

The release of glutamate is essential to the propagation of cortical SD (Van Harreveld and Fifkova 1973). Several studies have shown that glutamate acts via NMDA receptors (NMDARs) during the generation and propagation of SD (Mody et al. 1987; Gorelova et al. 1987; Marrannes et al. 1988; McLachlan 1992). Antagonists of NMDARs block SD in normal cortex, while non-NMDAR antagonists are ineffective (Anderson and Andrew 2002). The efficacy of NMDA antagonists to block SD is further supported by several studies in vivo (Lauritzen...
and Hansen 1992; Koroleva et al. 1998) and in vitro (Footitt and Newberry 1998; Somjen 2001).

At first, NMDA antagonists were considered as having great potential as neuroprotective agents (McCulloch 1994), but most clinical trials have been disappointing as the drugs caused psychomimetic and cardiovascular side effects severely limiting their usefulness (Menniti et al. 2000). Nevertheless, specific subunit-selective NMDA antagonists may still preserve neuroprotective properties without causing significant side effects (Menniti et al. 2000).

The NMDA receptor is a heterotetramer assembled from a NR1 subunits and at least one subtype of the four members of the NR2 (A-D) subunits family. The NR2A and NR2B subunits are predominantly expressed in the cortex and hippocampus (Monyer et al. 1992). Ifenprodil is part of a class of subunit-selective NMDA receptor antagonists with high selectivity for NR2B-containing receptors (Williams 1993). Its potency strongly depends on extracellular pH and is only weakly affected by NR1 subunit splice variants (Pahk and Williams 1997). A detailed characterization of the pharmacological properties of ifenprodil has been published (Grimwood et al. 2000).

Based on the role of NMDA receptors in the initiation and propagation of SD (Mody et al. 1987), the aim of our study was to identify possible subunit specificity in the process of SD generation in the entorhinal cortex. Our findings are consistent with a critical role of the NR2B subunits in the generation of entorhinal cortical SD.
Methods

Animals

The experiments were performed on 22 adult (2-3 months old) male mice (Harlan, San Diego, CA and Charles River Laboratories, Wilmington, MA). All experiments were carried out in accordance with a protocol approved by the UC Chancellors ARC conforming to the National Institute of Health (NIH) guidelines on the ethical use of animals in research. Only the minimum number of animals necessary to produce reliable scientific data was used in this study.

Slice preparation

Horizontal slices containing the entorhinal cortex and the hippocampus were obtained using standard technique (Gordey et al. 2001). Briefly, mice were anesthetized with halothane and decapitated. The brains were cooled to 4°C, rapidly removed and whole brain slices (350 µm thick) were cut in the horizontal plane on a Leica VT100S vibroslicer (Leica Microsystems, Deerfield, IL). After incubating in artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 2.0 mM CaCl$_2$, 2.0 mM MgCl$_2$, 26 mM NaHCO$_3$, and 10 mM D-glucose) for at least 1 hr in a storage chamber at 32°C, the slices were transferred to another chamber containing low Mg (0.1 mM MgCl$_2$); picrotoxin 50 µM; glycine 10 µM and 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) 10 µM,
designed to isolate NMDA field potentials (Crepel et al. 1997; Gordey et al. 2001; Dalby and Mody 2003). After 10-15 min, slices were transferred to a recording chamber and continually perfused with the latter ACSF (2 ml/min, 34°C) in a atmosphere of humidified 95% O₂, 5% CO₂.

**Extracellular Recordings**

Evoked extracellular fEPSPs were recorded in layers 2-3 of the entorhinal cortex. Constant current stimuli were delivered to layer 6 of the entorhinal cortex every 15 – 90 s via bipolar tungsten stimulating electrodes (Fig I.A). NMDAR-mediated fEPSPs were isolated in the presence of low Mg²⁺, 10 µM DNQX and 50 µM picrotoxin (Gordey et al. 2001). Field potential recordings started after 10 min of equilibration in the recording chamber. The amplitudes of NMDA receptor-mediated fEPSPs were determined as the difference between the baseline and the peak. The area was measured from the start of the downward deflection of the fEPSP until the point when the field potential returned back to the pre-stimulus baseline. For each experiment, stimulation intensity was chosen to generate a standard amplitude (0.7 – 1.0 mV) NMDA receptor-mediated field potential.

In our initial experiments, in order to obtain input-output curves, we changed the widths of the stimuli, while leaving the stimulus intensity at a fixed current level. The stimulus widths ranged from 20 to 240 µs at intensities of 30-35 mA. Longer stimulus widths (140 – 240 µs) led to the appearance of SDs. In
5 input-output curves done in control ACSF, SDs were induced 3 times with the stimulus widths of 140, 160 and 240 µs respectively. Therefore, in the experiments presented here we used the stimuli had widths between 40 - 60 µs.

Amplitude, duration and time to SD were also measured. The amplitude of the SD peak was determined as the difference between the baseline and the peak (as measured in fEPSPs). The duration of a single SD was measured as the time elapsed between the start of the negative deflection from baseline until the return of the DC filed potential to the same baseline. The time to SD occurrence was measured from the time a stable extracellular response was obtained until the first SD was recorded. We considered as an arbitrary cutoff time for establishing the absence of SDs a period of 40 min. This time corresponds to the average time of occurrence of SDs in control ACSF + 2 standard deviations.

**Data sampling**

Field potentials were amplified 100-fold through the headstage, and another 2-times through a BrownLee 440 Precision Amplifier (San Jose, CA) and low-pass filtered (DC – 3 kHz, 4 pole Bessel) before storing them on videotape (Neurocorder model PCM digitization at 88 kHz). Off-line, the recordings were replayed and low-pass filtered at DC – 300 Hz (4 pole Bessel) before digitized (at 1 kHz) and stored on a Pentium-processor-based PC using the in-house designed LabView event detection and analysis (EVAN) software package.
Drugs

Stock solutions of 100 mM DNQX (Tocris, Ellisville, MO) and 100 mM ifenprodil (Sigma, St. Louis, MO) were prepared in 100% dimethylsulfoxide (DMSO). For picrotoxin (Sigma), a 50 mM stock solution was prepared in ethanol (95% v/v). DMSO or ethanol alone in concentrations up to two-times higher than in our experimental conditions did not alter NMDA receptor-mediated fEPSPs.

Results

Properties of NMDA receptor-mediated fEPSPs

In the presence of a low Mg\(^{2+}\) ACSF containing DNQX and picrotoxin, we recorded typical NMDAR-mediated fEPSPs much like those observed in earlier studies in the hippocampus and dentate gyrus (Gordey et al. 2001; Dalby and Mody 2003). As shown in Table 1, the average peak amplitude of the NMDAR-mediated fEPSPs was 1.42 ± 0.58 mV (n=15) and their area was 944.1 ± 584.4 mV.ms (n=15). Perfusion of ifenprodil (10 µM) did not change the properties of NMDA fEPSP (fig.1 - B and Table 1).

In three experiments we compared the properties of NMDA fEPSPs before and after perfusion of 10 µM ifenprodil. Values of area, peak and decay time constants were measured in an average of at least 3 traces (evoked every 15 s) in the control solution and after 10 min after the start of ifenprodil perfusion and
were found not to be significantly different between the two conditions. The areas of fEPSPs in control and in ifenprodil solution were respectively (mean ± STD, in mV.ms): 399.3 ± 56.5 and 424.7 ± 145.7 (p = 0.31, paired t-test), the peak amplitudes were (in mV): 0.78 ± 0.27 and 0.80 ± 0.28 (p = 0.44, paired t-test) and the decay time constants were (in ms): 597.9 ± 79.5 and 536.5 ± 78.5 (p= 0.56, paired t-test). These findings, together with the group data presented in Table 1, indicate the lack of effect of 10 µM ifenprodil on the evoked NMDA fEPSPs, consistent with their mediation by NR2A containing receptors.

*Induction of SD in isolated NMDAR-mediated field potentials*

While recording fEPSP mediated by NMDARs, we noticed that spontaneous SDs developed within a certain time of the recordings. SDs were observed in 11 of 15 slices (in as many mice; frequency: 73.3%), at an average time of 18.5 ± 11.6 min after the start of the recordings, not counting the 10 min of equilibration time in the recording chamber. If a brain slice did not show SD after a recording period of 40 min, it was considered not to have developed any SD. In two experiments, we continued to record evoked responses beyond the 40 min cutoff time, and observed no SDs at 60 and 80 min respectively.

As noted by previous investigators, SDs are characterized by negative extracellular DC shifts reaching maxima of -30 mV and a duration of more than 0.5 - 1 min (Gorji 2001). In our experiments in layers 2-3 of the entorhinal cortex, the average amplitude of SD was 10.4 ± 4.4 mV while its duration was 51.6 ±
During the SD, the neurons of layers 2-3 were not responsive to electrical stimulation, as no fEPSPs could be elicited for approximately 2 min after the start of the SD. As shown in fig I. C-D, a stimulus delivered every 15 s could not evoke a response during the SD.

Frequently, more than 3 consecutives spontaneous SDs could be recorded showing the reversible nature of neuronal excitability after SD. The frequency of stimulation did not appear to be a factor in the time required to induce SD. In two slices, when the inter-stimulus interval was increased from 15 s to 90 s, SDs developed within 20 and 26 min respectively after the start of the stimulation.

**SD fails to develop in the presence of ifenprodil**

To address the specific contribution of NR2B receptors to the generation of SD, we examined the induction of spontaneous SDs in the entorhinal cortex in the presence of the NR2B-selective antagonist ifenprodil (10 µM). In contrast to our recordings without ifenprodil where SDs were induced in 11/15 slices, only one out of 7 slices developed SD in the presence of ifenprodil. In this slice SD occurred 7.4 min after the start of the recording, and had peak amplitude of -12.9 mV and duration of 51.7 s.

Comparing the proportion of slices developing SDs in the control and ifenprodil treated groups, the occurrence of SD was significantly lower in the
ifenprodil treated preparation ($\chi^2$ test; df = 1; $\chi^2 = 4.54$, P < 0.025). Interestingly, ifenprodil had no significant effect on the peak and area of NMDAR mediated fEPSP (two-way ANOVA, p > 0.05; Table 1).

**Discussion**

In the present study, we have recorded isolated fEPSPs mediated by NMDA receptors in layers 2-3 of the murine entorhinal cortex. Under our recording conditions with AMPA/KA and GABA\(_A\) receptors blocked, we have found no specific contribution of NR2B subunits to evoked fEPSPs. Nevertheless, these subunits were involved in the generation of SD in entorhinal cortical slices, as the induction of SD was nearly abolished in the presence of the NR2B subunit specific antagonist ifenprodil.

The properties of NMDA receptor-mediated fEPSPs in layers 2-3 of the entorhinal cortex recorded in the absence of GABA\(_A\), AMPA/KA receptors were similar in the presence of 10 \(\mu\)M ifenprodil, consistent with a lack of contribution of NR2B subunits to the evoked synaptic potentials. These findings were corroborated in experiments when the effect of ifenprodil was tested on NMDA fEPSPs of the same slices. In other brain regions, such as the hippocampal formation, experiments using similar ifenprodil concentrations have revealed a contribution of NR2B subunits to NMDA fEPSPs. In contrast to the responses recorded in layers 2-3 of the entorhinal cortex, isolated NMDA receptor-mediated fEPSPs in the dentate gyrus and CA1 region of the hippocampus (Gordey et al. 2001; Dalby and Mody 2003) had a reduced peak and a faster decay time.
constant in 10 µM ifenprodil indicating. Thus, the physiological mechanisms responsible for generating fEPSPs and possibly the localization of NR2B subunits at synapses are clearly different between layers 2-3 of the entorhinal cortex and the hippocampus.

Another difference between the entorhinal cortex and the hippocampus was the occurrence of SD. Previous studies using similar methods (Gordey et al. 2001; Dalby and Mody 2003) did not observe any SDs of isolated NMDA receptor-mediated synaptic transmission in the hippocampus. In contrast, spontaneous SDs were frequently observed in the layer 2-3 of entorhinal cortex when other ionotropic glutamate and GABA_A receptors were blocked. The amplitudes and durations of the SDs recorded under these conditions in the entorhinal cortex were not different than those recorded with intact AMPA/KA receptors (McLachlan and Girvin 1997), indicating that the NMDA receptors are critical in both the generation and in the shaping of SD waveforms. However, as both metabotropic glutamate and GABA_B receptors were intact in our experiments, these receptors may have also contributed to the development of SD. Our results using the specific NR2B receptor antagonist ifenprodil argue against this latter possibility. Our findings indicate that SDs generated in the entorhinal cortex critically depend on the activation of NMDA receptors containing ifenprodil-sensitive NR2B subunits. Previous studies with NMDA antagonists are in accordance with our present findings. The NR2B subunit antagonist, CP-101,606, a derivative of ifenprodil (Chenard et al. 1995) has been used to investigate electrically-induced cortical SD in rats in vivo (Menniti et al.)
Cortical SDs were inhibited by this compound in a dose-dependent fashion.

The results published by Somjen et al. (2000) validate the dual hypothesis of van Harreveld (1978) who suggested the existence of two kinds of SD: one mediated by glutamate and the other by $K^+$. The conclusion is that besides channels controlled by ionotropic glutamate receptors and TTX-sensitive $Na^+$ channels, other, as of yet unidentified, pathways can mediate SD-like depolarizations. Recent computer simulations performed by Kager et al. (2000; 2002), also suggest that more than one agent can induce SD, that various ion channels can provide the pathway for the ion fluxes underlying the depolarization. An increase in extracellular level of $([K^+]_o)$ and a change in glial buffering power, can influence the ability to generate SD.

The precise location of the NR2B subunits critically involved in the generation of SD remains to be determined. NR2B subunits appear to be present on presynaptic terminals of entorhinal cortical neurons in acutely prepared brain slices (Woodhall et al. 2001). Activation of these receptors may enhance glutamate release, facilitating the occurrence of SDs. Such presynaptic location of NR2B subunit-containing NMDA receptors would also be consistent with the lack of an NR2B receptor mediated component in the synaptic field potentials. Alternatively, predominantly extrasynaptically located NR2B subunit-containing NMDA receptors (Tovar and Westbrook 1999) could generate large depolarization when activated by ambient glutamate levels or when large glutamate overspill occurs. It is interesting to note that the time to the occurrence
of SD in our slices was independent of the frequency of stimulation, indicating that the amount of glutamate released from the stimulated fibers was not a key factor in the occurrence of SD. A study by Tovar and Westbrook (1999) in cell cultures showed predominantly synaptically localized NR2A receptors and an extrasynaptic spread of NR2B receptor subunits. We cannot exclude the possibility that in other layers of the entorhinal cortex NR2B subunit-containing NMDA receptors may participate in synaptic events, and those sites may be critically involved in the generation of SD.

Our studies have identified activation of NR2B subunit-containing NMDA receptors as being critical for the induction of SD in the entorhinal cortex. Notably, at therapeutically effective doses in clinical trials ifenprodil and its analogue eliprodil did not share the significant side effects such as ataxia and motor impairment of the non-subtype selective NMDA receptor antagonists (Chizh et al. 2001). Thus our results showing the dependence of cortical SD on NR2B subunit-containing NMDA receptor activation may open specific pharmacological approaches to the treatment of neurological disorders thought to involve SD.
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Figure Legends

Fig 1. Electrode positions and raw traces of fEPSPs and SDs. A. Schematic representation of a combined hippocampal/entorhinal cortical slice indicating the positions of the extracellular electrodes. The recording electrode was positioned in layers 2-3 while the stimulating electrode was placed in layer 6 of the entorhinal cortex. B. Representative averaged traces of evoked extracellular NMDA fEPSPs showing similar properties in control ACSF and after perfusion of a solution containing 10 µM ifenprodil. The bottom panel shows the two traces superimposed indicating no differences between the two averages. Vertical lines during the SDs represent stimulus artifacts that were ineffective in evoking field potentials. C. Representative trace of a SD event observed in trace was similar between both groups as can be seen in control (D) or in 11 of 15 slices under control conditions. D. Raw trace of the SD observed in only one of 7 slices in the presence of ifenprodil.
Table 1. Properties of fEPSPs and SDs observed in control ACSF and in the presence of ifenprodil (10 μM).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ifenprodil (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fEPSP Peak (mV)</td>
<td>1.42 ± 0.58</td>
<td>1.40 ± 0.61</td>
</tr>
<tr>
<td>fEPSP Area (mV.ms)</td>
<td>944.1 ± 584.4</td>
<td>860.4 ± 660.9</td>
</tr>
<tr>
<td>Number of mice (slices)</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>SD Peak (mV)</td>
<td>10.4 ± 4.4</td>
<td>12.9</td>
</tr>
<tr>
<td>SD Duration (s)</td>
<td>51.6 ± 29.3</td>
<td>51.7</td>
</tr>
<tr>
<td>Time to SD (min)</td>
<td>18.6 ± 11.6</td>
<td>7.4</td>
</tr>
<tr>
<td>Number of slices with SD</td>
<td>11</td>
<td>1 *</td>
</tr>
</tbody>
</table>

Recordings were done in a single slice obtained from each experimental animal. Numbers represent mean values ± SD. *Denotes statistically significant difference from control according to a χ² test; (df = 1; P <0.025).
Figure 1

A

B

Control

Ifenprodil 10μM

Superimposed

0.5mV
300ms

C

D

2mV
10s