Dizocilpine binding to cerebral cortical membranes from developing and ageing mice

Pirjo Saransaaria,*, Simo S. Ojaa, b

aDepartment of Physiology, Tampere Brain Research Center, University of Tampere School of Medicine, Box 607, FIN-33101 Tampere, Finland
bDepartment of Clinical Physiology, Tampere University Hospital, Box 2000, FIN-33521 Tampere, Finland

Received 12 May 1995: revised 2 September 1995; accepted 25 September 1995

Abstract

The binding of [$^3$H]dizocilpine (MK-801) to the N-methyl-D-aspartate (NMDA)-gated ion channel was characterized in cerebral cortical membranes during the major portion of the mouse life-span (from 7-day- to 22-month-olds). The binding was saturable, consisting of only one component at all ages studied. The maximal binding capacity $B_{\text{max}}$ was very substantial in 14-day-old mice when compared to adults (3-month-olds), decreasing thereafter during ageing. The binding constant $K_D$ remained unchanged during development and increased only slightly in aged mice. Glutamate and glycine potentiated dizocilpine binding concentration-dependently. Their efficacy varied markedly with age. Both glutamate and glycine had considerably less effect on the immature cerebral cortex and in the oldest group of mice (22-month-old) than in young adults. The marked increase in dizocilpine binding sites at the age of 2 weeks coincides with the previously reported transient increase in NMDA binding sites in the cerebral cortex. The weak potentiation of dizocilpine binding by glutamate and glycine in the immature brain could be a factor which protects neurons during this period from excitotoxicity and increased susceptibility to seizures induced by acidic amino acids. The decrease in the number of dizocilpine binding sites during ageing could result partly from the loss of cortical neurons.

* Corresponding author. Tel.: + 358 31 2156873; Fax: + 358 31 2156170.

0047-6374/95/$09.50 © 1995 Elsevier Science Ireland Ltd. All rights reserved
SSDI 0047-6374(95)01665-M
1. Introduction

During recent years, the N-methyl-D-aspartate (NMDA) class of glutamate receptors has been intensively investigated by electrophysiological, biochemical, pharmacological and molecular biological methods. It is recognized as a ligand-gated ion channel with complex regulation. The functional significance of the NMDA receptor complex is becoming more clear. It is a central component in the generation of long-term potentiation, a long-lasting enhancement of synaptic activity that underlies learning, memory and neuronal plasticity. These properties render the NMDA receptor essential for neuronal induction, proliferation and differentiation during the developmental period [1]. On the other hand, NMDA receptor functions are involved in ageing changes in the brain associated with impeding impairment of learning and memory [2]. Overstimulation of the NMDA receptor leads to an excessive influx of Ca\(^{2+}\), ultimately inducing neuronal death. This excitotoxic effect of glutamate has been thought to play a key role in neurodegenerative diseases and is also implicated in the loss of neurons during normal ageing [3].

The NMDA receptor-ionophore complex consists of at least four distinct binding sites, including (1) an NMDA recognition site with high-affinity for glutamate, (2) a glycine recognition site insensitive to strychnine, (3) a polyamine recognition site, and (4) an ion channel domain permeable to Ca\(^{2+}\) and Na\(^{+}\) [4]. The first three binding sites stimulate opening of the ion channel, whereas the channel itself possesses binding sites sensitive to inhibition by Mg\(^{2+}\), Zn\(^{2+}\), H\(^{+}\) and allosteric blockers such as dizocilpine (MK-801) and phencyclidine (PCP). Since the dizocilpine site is thought to lie within the cation channel in the receptor complex [5], binding of radioactive dizocilpine can be used as an index of the open state of the channel [6].

In order to better understand the fundamental roles of the NMDA receptor complex in developmental and ageing processes, we have recently characterized age-related alterations in the binding of \[^{3}H\]glycine [7] and \(N\)-[1-(2-thienyl)cyclohexyl][\(^{3}H\)piperidine (\[^{3}H\]TCP) [8], a ligand of the phencyclidine sites, to the receptor complex in the mouse cerebral cortex. Now we have extended this investigation to the functional properties of the receptor-gated channel by measuring the binding of \[^{3}H\]dizocilpine to cerebral cortical membranes during the major part of the mouse life-span.

2. Materials and methods

2.1. Materials

NMRI mice of both sexes from 7 days to 22 months of age were used in the experiments. \[^{3}H\]Dizocilpine (0.814 PBq/mol) was obtained from New England Nuclear, Boston, MA. Unlabeled dizocilpine was a gift from Merck, Sharp and Dohme, Rahway, NJ. Other reagents and drugs were from commercial sources.
Membranes for \[^{1}H\]dizocilpine-binding experiments were prepared from the cerebral cortices of mice of different ages as described by Procter et al. [9,10] with some modifications. The cerebral cortices were homogenized in nine volumes (wt/vol) of 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 20,000 x g for 20 min. The resulting pellet was suspended in 75 volumes of Tris buffer and centrifuged at 48,000 x g for 25 min. This washing was repeated once with Tris buffer and three times with water before the pellet was stored at −70°C for at least 18 h.

On the day of the binding experiments the membrane pellet was thawed at room temperature, suspended in 75 volumes of 5 mM Tris-HCl buffer (pH 7.4), centrifuged at 48,000 x g for 30 min and then subjected three more times to this washing. In the binding assays membrane preparations (about 0.2–0.3 mg protein) were incubated in Tris-HCl buffer with \[^{3}H\]dizocilpine at 25°C for 45 min. The incubation was stopped by rapid filtration on Whatman GF/B filters previously soaked in a 0.1% solution of polyethyleneimine. The filters were rinsed twice with 5 ml of ice-cold buffer. The radioactivity trapped in the filters was measured by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 25 μM dizocilpine. The protein concentration was measured by the method of Lowry et al. [11].

2.3. Calculations

The kinetics of the binding were assessed within the concentration range of 0.5–200 nM and the parameters (± SEM) estimated by nonlinear regression analysis. The fitting was done iteratively with the simplex algorithm [12]. The results were subjected to analysis of variance, followed by Hartley’s sequential method of testing individual differences.

3. Results

The specific binding of \[^{3}H\]dizocilpine to cerebral cortical membranes was about 90% of the total binding at all ages studied. The binding was saturable (Fig. 1A and Fig. 2A), consisting of only one binding component, as revealed by the constructed Scatchard plots (Fig. 1B and Fig. 2B). The amount of binding was greatest in 14-day-old mice (Fig. 1), followed by 6-month-old mice (Fig. 2). The calculated maximal binding capacity \(B_{\text{max}}\) was markedly greater in 14-day-old mice than in 7-day-olds and young, 3-month-old adults (Table 1). Already at 6 months of age, \(B_{\text{max}}\) had been decreased almost to the same level as in 7-day-old animals. The binding constant \(K_{D}\) remained unchanged during development but was significantly higher in the oldest groups of mice than in the 3-month-olds.

Both glycine and glutamate potentiated dizocilpine binding concentration-dependently, the efficacy of the stimulation varying with age (Fig. 3). The EC\textsubscript{50} value of
concentration nM

bound

binding µmol/kg protein

0.20

0.16

0.12

0.08

0.04

0.04

0.08

0.12

0.16

0.20

0.20

0.16

0.12

0.08

0.04

0.04

0.08

0.12

0.16

0.20

Fig. 1. (A) Concentration dependence of dizocilpine binding to cerebral cortical membranes from 7-day-old (••••), 14-day-old (△△△△) and 3-month-old (●●●●) mice. The results are mean values (± SEM) from 4 separate experiments. (B) Scatchard plots of the same data. The calculated binding parameters are compiled in Table 1.

glutamate was significantly smaller in the membranes of 7-day-old and greater in 18-month-old mice than at the other ages studied (Table 2). The maximal stimulation was small (about 60%) in 14-day-olds and also markedly reduced during ageing (Fig. 3, Table 2). The maximal potentiation of dizocilpine binding by glycine was also very small (about 30%) in membranes from 14-day-olds and also reduced in old mice (Fig. 3, Table 2). The EC₅₀ value for glycine was significantly smaller in 7-day-old mice than in adults. The effect of glutamate and glycine together (both 10 μM) was greatest in 7-day-olds and smallest in 14-day-olds (Table 3), the stimulations being not additive, however. NMDA (10 μM) also potentiated dizocilpine binding, the effect being lowest in 14-day-olds and aged animals (Table 3). The binding was unaffected by strychnine (10 μM) but moderately inhibited by 3-amino-1-hydroxypropylidin-2-one (HA-966, 10 μM) and very strongly by 7-chlorokynurenate (Table 3). The inhibitions were generally most pronounced in 3-month-olds. Moreover, the binding was not influenced by taurine or β-alanine (10 μM to 1.0 mM) (Table 3). Nor were the stimulations by glutamate or glycine affected by taurine (1.0 mM) (data not shown).

4. Discussion

The general characteristics of dizocilpine binding in the mouse cerebral cortex in the present study were similar to those previously reported for different brain
regions of rodents and humans. For example, the binding constant \( (K_D) \) is also of same order of magnitude in the cat, monkey and rat cerebral cortex \([13,14]\) and rat forebrain \([15]\), when binding is measured without simulators. Glutamate and glycine potentiate binding by rendering the ion channel more accessible to dizocilpine \([4]\), and slightly diminish \( K_D \) \([16–19]\). The maximal binding capacity \( (B_{\text{max}}) \) in the mouse cerebral cortex was also fairly similar to that in the brains of rodents and humans \([14,16,18,19]\). Developmental and ageing processes seemed to

Table 1
Parameters of dizocilpine binding to mouse cerebral cortical membranes

<table>
<thead>
<tr>
<th>Age</th>
<th>( K_D ) (nM)</th>
<th>( B_{\text{max}} ) (μmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td>30.0±2.4</td>
<td>2.47±0.11 * *</td>
</tr>
<tr>
<td>14 days</td>
<td>33.6±3.4</td>
<td>5.12±0.15 * *</td>
</tr>
<tr>
<td>3 months</td>
<td>30.3±1.6</td>
<td>3.59±0.05</td>
</tr>
<tr>
<td>6 months</td>
<td>34.8±3.0</td>
<td>4.11±0.10 * *</td>
</tr>
<tr>
<td>12 months</td>
<td>35.7±2.0</td>
<td>2.90±0.05 * *</td>
</tr>
<tr>
<td>18 months</td>
<td>40.7±2.7 * *</td>
<td>3.13±0.07 * *</td>
</tr>
<tr>
<td>22 months</td>
<td>38.0±3.8 *</td>
<td>2.92±0.09 * *</td>
</tr>
</tbody>
</table>

The parameters (±SEM) were estimated by nonlinear regression analysis using the simplex algorithm. Significance of the differences compared to 3-month-olds: \* \( P<0.05 \); \* \* \( P<0.01 \).
Fig. 3. Potentiation of dizocilpine binding by glutamate (-o-) or glycine (-•-) to cerebral cortical membranes isolated from (A) 7-day-, (B) 14-day-, (C) 3-month-, (D) 18-month- and (E) 22-month-old mice. The results are mean values (± SEM) of 4-8 separate experiments carried out at 6 different concentrations of glutamate or glycine. The dizocilpine concentration was 5 nM. The calculated ED₅₀ values are compiled in Table 2.

A rapid increment of the brain weight, intense formation of synapses and proliferation of axons in the cerebral cortex are salient features of the early postnatal development in rodents [20,21]. During the postnatal week the number of axons per unit volume density actually exceed the final adult density in the cortex [22]. Thereafter brain growth slows down and the brain weight in relation to the body weight begins to decline markedly [20,21]. The pronounced enhancement of dizocilpine binding at the age of two weeks also coincides with the transient increase in the number of NMDA binding sites observed in the rat [19,23-25] and human cerebral cortex and hippocampus [26-29]. A similar increase has been found in the number of glutamate binding sites [30] and phencyclidine sites in the NMDA receptor [8,30], indicating that the binding domains in the cation channel and agonist site in the NMDA receptor complex have the same developmental time...
Table 2
Potentiation of dizocilpine binding to mouse cerebral cortical membranes by glutamate and glycine

<table>
<thead>
<tr>
<th>Age</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Maximal stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glutamate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>66.8 (63.0–71.0)*</td>
<td>192.9 ± 2.9</td>
</tr>
<tr>
<td>14 days</td>
<td>128.9 (121.2–137.5)</td>
<td>62.7 ± 1.0*</td>
</tr>
<tr>
<td>3 months</td>
<td>142.7 (136.3–149.6)</td>
<td>202.9 ± 2.5</td>
</tr>
<tr>
<td>18 months</td>
<td>196.7 (177.6–219.7)*</td>
<td>135.2 ± 3.0*</td>
</tr>
<tr>
<td>22 months</td>
<td>147.3 (125.1–177.2)</td>
<td>99.1 ± 4.3*</td>
</tr>
<tr>
<td><strong>Glycine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>113.6 (94.0–141.5)*</td>
<td>121.3 ± 5.8*</td>
</tr>
<tr>
<td>14 days</td>
<td>190.7 (153.5–246.8)*</td>
<td>28.5 ± 1.7*</td>
</tr>
<tr>
<td>3 months</td>
<td>277.5 (251.7–308.1)</td>
<td>152.4 ± 3.9</td>
</tr>
<tr>
<td>18 months</td>
<td>234.4 (219.3–251.5)</td>
<td>110.6 ± 2.0*</td>
</tr>
<tr>
<td>22 months</td>
<td>193.1 (171.6–219.7)*</td>
<td>41.0 ± 1.4*</td>
</tr>
</tbody>
</table>

The results are means ± SEM (n = 3–4). The dizocilpine concentration was 5 nM. The experiments were carried out using 6 different concentrations of glutamate or glycine.

Significance of differences when compared to 3-month-olds: ** * * P < 0.01. * P < 0.05.

Table 3
Modification of dizocilpine binding to mouse cerebral cortical membranes

<table>
<thead>
<tr>
<th>Effector</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days</td>
</tr>
<tr>
<td>glu + gly</td>
<td>350.9 ± 25.5*</td>
</tr>
<tr>
<td>NMDA</td>
<td>211.2 ± 7.1*</td>
</tr>
<tr>
<td>HA-966</td>
<td>66.6 ± 1.3*</td>
</tr>
<tr>
<td>7-Cl-kyn</td>
<td>34.7 ± 0.8*</td>
</tr>
<tr>
<td>Strychnine</td>
<td>85.5 ± 8.1</td>
</tr>
<tr>
<td>Taurine</td>
<td>105.9 ± 6.2</td>
</tr>
<tr>
<td>β-alanine</td>
<td>92.9 ± 5.1</td>
</tr>
</tbody>
</table>

The results are means (± SEM) of 3 separate experiments. The dizocilpine concentration was 5 nM and the concentrations of the effectors 10 μM.

Significance of differences compared to 3-month-olds: * * * P < 0.01.

Abbreviation: 7-Cl-kyn, 7-Cl-kynurenate.

course. Molecular biochemical experiments have further shown that in the frontal cortex of developing rats, NMDA-R1 mRNA, which codes the major component of the ion channel in the NMDA receptor complex [31], is increased almost threefold from postnatal day 3 to day 15 [32].

Concomitantly with the transient increase in the number of dizocilpine binding sites, the maximal potentiations by glutamate and glycine were markedly reduced,
indicating that the functions of the receptor itself are also altered. On the other hand, the glutamate content markedly increases in the mouse cerebral cortex during the two first postnatal weeks [33]. In keeping with the present findings, the maximal stimulation of dizocilpine binding by glycine is lower in the immature than mature rat forebrain, though without changes in the EC₅₀ values between 8 days of age and adulthood [25]. Strychnine-insensitive glycine binding is also maximal in the mouse cerebral cortex [7] and rat forebrain [34] at 2 weeks of age. During the first weeks of life in rodents, postsynaptic excitation predominates over inhibition. The neocortical networks in rats then exhibit strong NMDA-receptor-mediated polysynaptic interactions [35]. The early developmental overexpression of NMDA receptor channel activity also coincides with an increased susceptibility to seizures and excitotoxicity produced by acidic amino acid analogs [1]. The decreased potentiation by extracellular glutamate and glycine during that period could thus protect neurons from damage.

The significant reduction in the density of dizocilpine binding sites in the cerebral cortex of the aged mouse is consistent with previous findings of an age-related decrease in the number of NMDA receptors in various brain areas in rodents and humans [15–18,25,36–38]. The decrease may result from neuronal loss and dendritic atrophy during ageing [39], even though in mice the age-related changes in neuronal number are not so impressive as in some other animals species [40] and the brain weight even increases during ageing [41]. The age-related reduction in NMDA binding does not appear to be attributable solely to an increase in membrane protein, as suggested by Bonhaus et al. [37], although the protein content of membranes was also now slightly greater in aged than in young adult mice. In addition to the reduced number of dizocilpine binding sites the binding affinity was decreased as well, similarly to the phencyclidine binding sites in the cerebral cortex of aged mice [8].

The age-related decline in cognition and memory is a well-known phenomenon in man and animals [42,43]. Ageing is also known to be associated with deficits in neuronal plasticity, demonstrated, for example, with rats learning spatial tasks such as the Morris water maze [44,45] and in kindling experiments [46]. The neuronal plasticity required in learning spatial tasks and development of kindling depends upon excitatory amino acid neurotransmission mediated by NMDA receptors. NMDA antagonists such as dizocilpine reduce the rate of learning [47–49]. A possible correlation seems to prevail between the reduction in NMDA receptor density and the cognitive decline in ageing [50]. Our observation of a decrease in dizocilpine binding sites thus corroborates the proposition that the principal defect underlying reduced neuronal plasticity in aged rats may be an impairment in NMDA receptor-mediated neurotransmission.

The reduction in the number of dizocilpine binding sites does not appear to be the only alteration in the NMDA receptor complex induced by ageing, since the potentiation of dizocilpine binding by both glutamate and glycine was also decreased. Receptor modulation may thus be impaired. Changes during ageing could involve not only a reduction in the number of cell bodies containing NMDA receptors but also a downregulation of the NMDA receptor-governed ion channel.
A reduction in glycine stimulation of dizocilpine binding in the presence of glutamate has been reported in Alzheimer's disease [9]. On the other hand, a compensatory increase in the sensitivity of dizocilpine binding to glutamate and glycine has been observed in the hippocampus in aged rats [18]. Furthermore, increased strychnine-insensitive glycine binding in aged mice may also partly compensate for the decreased efficacy of glycine [7].

Neurons with NMDA receptors are vulnerable to excitotoxic injury caused by an excess of extracellular glutamate. An age-related increase in glutamate release has been reported in the cerebral cortex [33], together with a reduced uptake [33,51]. The extracellular levels of glutamate are thus elevated in aged subjects. The age-related reduction in the number and functional efficacy of NMDA receptors may represent a compensatory downregulation to protect neurons bearing NMDA receptors from excitotoxic insults caused by increased concentrations of extracellular glutamate, though at the expense of an impairment of their functional efficacy.

Acknowledgements

The skilful technical assistance of Mrs Oili Pääkkönen and the financial support of the Medical Research Fund of Tampere University Hospital are gratefully acknowledged.

References


