NMDA Receptor-Mediated Differential Laminar Susceptibility to the Intracellular Ca\(^{2+}\) Accumulation Induced by Oxygen-Glucose Deprivation in Rat Neocortical Slices

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Fukuda, Atsuo, Kanji Muramatsu, Akihito Okabe, Yasunobu Shimano, Hideki Hida, Ichiro Fujimoto, and Hitoo Nishino. NMDA receptor-mediated differential laminar susceptibility to the intracellular Ca\(^{2+}\) accumulation induced by oxygen-glucose deprivation in rat neocortical slices. J. Neurophysiol. 79: 430–438, 1998. Slices of somatosensory cortex taken from immature rats on postnatal day (P)7–14 were labeled with fura-2. Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was monitored in identified pyramidal cells as the ratio of fluorescence intensities (R\(_{340/380}\)) during oxygen-glucose deprivation. The R\(_{340/380}\) ([Ca\(^{2+}\)]\(_i\)) of individual pyramidal cells was monitored in each of the cortical layers II–VI simultaneously. Neurons in all neocortical layers exhibited significant increases in [Ca\(^{2+}\)]\(_i\), that varied with the duration of oxygen-glucose deprivation. Individual neurons responded to oxygen-glucose deprivation with abrupt increases in [Ca\(^{2+}\)]\(_i\), after various latencies. The ceiling level of the [Ca\(^{2+}\)]\(_i\), increase differed from cell to cell. Neurons in layer II/III showed significantly greater increases in [Ca\(^{2+}\)]\(_i\) than those in layers IV, V, or VI. Kynurenic acid, a nonselective glutamate receptor antagonist, and bicuculline, a selective \(\gamma\)-aminobutyric acid (GABA)\(_A\) receptor antagonist, suppressed the intracellular Ca\(^{2+}\) accumulation induced by oxygen-glucose deprivation in all neocortical layers examined. After kynurenic acid, but not bicuculline, there was no longer a differential [Ca\(^{2+}\)]\(_i\) increase in layer II/III. Both 2-amino-5-phosphono-pentanoic acid (AP5), a selective N-methyl-D-aspartate (NMDA) receptor antagonist, and 6-cyano-7-nitroquinodimine-2,3-dione (CNQX), a non-NMDA receptor antagonist, strongly suppressed the intracellular Ca\(^{2+}\) accumulation induced by oxygen-glucose deprivation in all layers. The laminar difference in terms of the [Ca\(^{2+}\)]\(_i\), increases was abolished by AP5, but not by CNQX. These results indicate that layer II/III cells are the most prone to oxygen-glucose deprivation-induced intracellular Ca\(^{2+}\) accumulation, and that this is primarily mediated by NMDA receptors. Thus, layer II/III neurons would be more likely to suffer cellular Ca\(^{2+}\) overload and excitotoxicity during ischemia than layer IV–VI cells. Such a differential laminar vulnerability might play an important role in determining the pathological characteristics of the immature cortex and its sequelae later in life.

INTRODUCTION

The neocortex is one of the regions of the brain most vulnerable to ischemia (Pulsinelli 1985). Among the subgroups of neurons in the neocortex, pyramidal neurons are thought to be the most vulnerable to ischemia and/or NMDA excitotoxicity (Beal et al. 1991; Freund et al. 1990). A large number of studies, most of which were performed on cultured neocortical neurons, have disclosed that an NMDA receptor-mediated [Ca\(^{2+}\)] increase plays a critical role in the excitotoxic cell death that can occur in the event of neocortical hypoxia and ischemia (see Choi 1990 for review). However, it has not been established whether regional differences exist in the neocortex’s vulnerability to Ca\(^{2+}\) overload. Although several studies have been carried out on neocortical slices to observe the effects of hypoxia/ischemia, including the resulting [Ca\(^{2+}\)], increase (Bickler et al. 1993; Bickler and Hansen 1994; Duffy and MacVicar 1996; O’Donnell and Bickler 1994), no studies have yet addressed the question of whether there are laminar differences in intracellular Ca\(^{2+}\) accumulation.

It is known that there are laminar differences in the susceptibility of neurons to NMDA-induced [Ca\(^{2+}\)], increases in the visual cortex (Lin et al. 1994). Previous reports have shown that GABAergic inhibitory systems strongly regulate the NMDA component of the glutamatergic excitatory postsynaptic potential in the neocortex while depression of GABAergic inhibition results in an accentuation of NMDA receptor-mediated excitatory postsynaptic potentials (Kanter et al. 1996; Luhmann and Prince 1990a,b; Metherate and Ashe 1994, 1995). During ischemia, the levels of both glutamate and GABA increase in the extracellular space in the cortex (Matsumoto et al. 1996; O’Regan et al. 1995). Thus, if there are topographical differences in the response to these agonists during ischemia, laminar differences in the vulnerability to Ca\(^{2+}\) overload might also exist. For this reason, we addressed the question of whether the neocortex shows a laminar difference in the degree of ischemia-induced intracellular Ca\(^{2+}\) accumulation with special reference to any differential effects mediated via glutamatergic and GABAergic receptors.

In the present study, we recorded [Ca\(^{2+}\)], transients during oxygen-glucose deprivation in neocortical slices from immature (P7–14) rats. The results show that [Ca\(^{2+}\)], increased during oxygen-glucose deprivation in layers II/III-VI, with the increase in layer II/III being the greatest. This laminar difference was abolished by AP5 or kynurenic acid, but not by CNQX or bicuculline. The data suggest that layer II/III cells are the cells most likely to experience oxygen-glucose deprivation-induced [Ca\(^{2+}\)], increases through NMDA receptor mediation. Some of these results have been published in abstract form (Fukuda et al. 1996b).

METHODS

The techniques used for preparing and maintaining neocortical thin slices in vitro and for obtaining optical recordings were similar...
to those described previously (Fukuda et al. 1995, 1996a). Wistar rats aged P7–14 were anesthetized with pentobarbital (50 mg/kg ip) and decapitated. The protocols used conformed with guidelines on the conduct of animal experiments issued by Nagoya City University. A block of the brain including the neocortex was quickly removed and placed in cold (4°C), oxygenated, modified artificial cerebrospinal fluid (ACSF), in which sucrose was substituted for NaCl (Aghajanian and Rasmussen 1989). CaCl2 was reduced, and MgSO4 was increased (Fukuda and Prince 1992a). This modified ACSF is known to yield healthier slices (Fukuda and Prince 1992a). The solution contained the following (in mM): 230 sucrose, 2.5 KCl, 1.25 NaH2PO4, 10.0 MgSO4, 0.5 CaCl2, 26 NaHCO3, and 30 glucose. Coronal slices of somatosensory cortex with a thickness of 150 μm were cut in the modified ACSF using a vibratome (DTK-1500, Dosaka). Slices were allowed to recover for 60 min on nylon meshes (with 100 μm pores) which were placed on culture dishes and submerged in standard ACSF consisting of (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2.0 MgSO4, 2.0 CaCl2, 26 NaHCO3, and 20 glucose. The culture dishes were placed in a tightly sealed box filled with 95% O2-5% CO2 at a pressure of 50 kPa at room temperature. Neurons were then loaded with the calcium indicator, fura-2, by incubating slices with fura-2 acetoxyl methyl (AM, 10 μM) in standard ACSF containing 0.0125% pluronic F127 for 60–90 min under the conditions described above, followed by washing with standard ACSF. To reduce background fluorescence, fura-2 was loaded into cells on the upper surface only. This was achieved by placing slices with the bottom surface (i.e., unloaded surface) against the petri dish (i.e., without nylon mesh). Slices were then laid on the glass bottom of a submersed type chamber that was placed on a microscope stage and continuously perfused with standard ACSF gassed with 95% O2-5% CO2 at a rate of 3–4 ml/min. The bathing solution, which was maintained at a temperature of 22–24°C, had a pH of 7.4 when saturated with 95% O2-5% CO2. Oxygen-glucose deprivation was achieved by switching from this solution to glucose-free ACSF saturated with 95% N2-5% CO2 (pH 7.4).

The fluorescence of fura-2 was excited by means of a xenon arc lamp (75W) and the emitted light was filtered with a band-pass filter (490–510 nm). Fluorescent images were obtained via a silicon intensifier target (SIT) camera (C2400, Hamamatsu Photonics) fitted to an upright microscope (Axiioskop FS, modified to be UV light compatible, Zeiss). Images were digitized on-line and 128 frames were averaged to improve the signal-to-noise ratio; they were then stored on magneto-optical disks. Data were analyzed offline on a computer with an image processor and data-analysis software (Argus-50, Hamamatsu Photonics). [Ca2+]i was expressed as the ratio of the fura-2 fluorescence intensity excited at 340 ± 5 nm to that excited at 380 ± 5 nm (RF340/F380). The apparent bleaching of fura-2, as indicated by a decrease in F360, was <6% after a 50 min experimental period, using the same recording protocol as for F340/F380. Thus, bleaching is not considered to be significant, and any influence it had would be canceled out by calculating the ratio F340 to F380. The RF340/F380 was monitored in each neocortical layer (i.e., II/III, IV, V, and VI) every 2 min before and during oxygen-glucose deprivation. Since we were aware that there is some absorption of 340 nm light by higher doses of kynurenic acid (0.5 mM), we did not attempt to calculate absolute [Ca2+]i. A normalized RF340/F380 was calculated for the temporal analysis of [Ca2+]i changes: each RF340/F380 value was divided by the baseline RF340/F380 (obtained by averaging 3 values obtained before the initiation of oxygen-glucose deprivation). Thus, changes in [Ca2+]i could be assessed over time in single cells in any of the different layers. The average baseline drift over a 50 min period of perfusion with ACSF was <3% for all layers. Shifts in the resting RF340/F380 in the presence of kynurenic acid, CNQX, and AP5 were <3% (but for bicuculline see Fig. 3 in the accompanying paper, Fukuda et al. 1998). Nomarski differential interference contrast images and optical recordings were obtained using a ×10 objective lens (Zeiss, Plan-NEOFLUAR, 0.3 N.A.) which allowed simultaneous observation of layers II through VI of the somatosensory cortex of the immature rat (Fig. 1, A–C). A number of living cells, with their membrane exposed, was located at the upper surface of each slice. Therefore, the focus plane was set for these cells for imaging purposes. With Nomarski images magnified digitally off-line, it was possible to distinguish living pyramidal neurons by their normal morphological features (Fig. 1D). Calculation of RF340/F380 was attempted only in pyramidal neurons identified off-line in layers II–VI.

All experiments were performed within 4 h after loading fura-2, because laminar differences in the emergence of deteriorated cells were noted in cortical slices that had been maintained under physiological conditions for more than 6 h (Fukuda et al. 1995). Throughout this report, results are expressed as means ± SD unless otherwise indicated. The following drugs were used: fura-2 AM, dimethylsulfoxide (DMSO), and pluronic F127 (Molecular Probes); (-)-bicuculline methiodide and kynurenic acid (Sigma). DL-AP5 and CNQX (Tocris Cookson). A 10 mM stock solution of fura-2 AM in 100% dry DMSO was mixed with 25% pluronic F127 in DMSO and added to the ACSF to yield a final concentration of 10 μM (0.1% DMSO and 0.0125% pluronic F127). Bicuculline (10 μM), kynurenic acid (500 μM), DL-AP5 (20 μM), and CNQX (10 μM) were applied by bath perfusion in certain experiments.

**RESULTS**

**Definition of cortical layers and identification of pyramidal neurons**

Slices were viewed under a microscope with Nomarski optics after labeling with fura-2. The cortical structure was recognizable, and it was possible to determine the borders between the layers of the somatosensory cortex by reference to the published criteria (Zilles and Wree 1994) and on the basis of both Nomarski and fluorescence images (Fig. 1, A–C). Pyramidal cells could be identified by their morphological characteristics (i.e., pyramidal-shaped cell body and apical dendrites ascending toward the pial surface; Fig. 1D). After identification, corresponding fluorescence images and ratio-images were selected for analysis (Fig. 1, E and F). Ratio-images were offset according to fluorescence intensity, so that fluorescence data from, for instance, cells insufficiently loaded with fura-2, unfocused and/or unidentified cells, and other background fluorescence (probably including glial cells) were excluded from the analysis.

**Oxygen-glucose deprivation increased [Ca2+]i in pyramidal neurons in all layers**

Before the initiation of oxygen-glucose deprivation, the resting RF340/F380 values obtained from identified pyramidal cells at room temperature were as follows (n = 140 in each layer from a total of 20 slices): layer II/III, 0.87 ± 0.14; layer IV, 0.87 ± 0.14; layer V, 0.86 ± 0.16; layer VI, 0.84 ± 0.21 (Fig. 2, A and B). There was no significant difference among layers in terms of the resting RF340/F380 (P > 0.4, one-way ANOVA), indicating that the resting level of [Ca2+]i in pyramidal neurons probably does not differ by layer. Subsequently, a 45 min period of oxygen-glucose deprivation evoked accumulations (>10%) of intracellular Ca2+ in cortical pyramidal neurons in all layers. The percentage of cells

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FIG. 1. Labeling with fura-2 and identification of pyramidal neurons in thin slice from immature neocortex. A: schematic diagram of a neocortical slice with indication of borders between layers. Grayed area corresponds to the viewed area, which is imaged below. B: neurons in slices were identifiable with the aid of Nomarski optics, thus laminar borders could be defined. Nylon threads used to hold the slice are visible at both edges of the view. C: the same slice as in B. Fluorescence emission at 510 nm excited by 380 nm was detected with an SIT camera. The slice was incubated with fura-2, AM (10 μM) for 90 min. Bars in B and C, 200 μm. D: the same image as in B digitally magnified so that individual pyramidal cells are recognizable. Arrows and arrowheads indicate apparent pyramidal neurons. E: a digital magnification of C that coincides with the area shown in D. Arrows and arrowheads correspond to those shown in D. F: a ratio-image produced by calculation of R F340/F380 by the image processor. The image was magnified so as to match the area of the Nomarski (D) and fluorescence (E) images. Note that cells insufficiently loaded with fura-2 cells are offset (arrowheads). This kind of cell was excluded from the analysis. Arrows and arrowheads correspond to those shown in D and E. Bars in D–F, 40 μm. R F340/F380 is used as a [Ca²⁺] indicator throughout this report.

analyzed that showed such an accumulation was layer II/III, 98%; layer IV, 96%; layer V, 94%; layer VI, 89% (Fig. 2, A and B). Observation of temporal changes in [Ca²⁺], in individual cells revealed that more or less abrupt increases in [Ca²⁺], occurred with latencies (measured to the establishment of a continuous increase of >10% from baseline) varying from 3 to 40 min after the beginning of oxygen-glucose deprivation. The latencies were (in min) layer II/III, 11.3 ± 7.7, n = 137; layer IV, 16.0 ± 9.6, n = 135; layer V, 18.5 ± 11.4, n = 132; layer VI, 20.0 ± 11.6, n = 125 (Fig. 2C). The latencies measured for cells in layer II/III were significantly shorter than those measured for cells in the other layers (P < 0.01, one-way ANOVA followed by Tukey-Kramer test).

Differential increases in [Ca²⁺], according to neocortical layer during oxygen-glucose deprivation

The normalized R F340/F380 values increased during oxygen-glucose deprivation in all layers. At this point, it should be mentioned that the ratio F340/F380 measured using fura-2 may be less sensitive to Ca²⁺ at higher Ca²⁺ concentrations, leading to a degree of nonlinearity. Nevertheless, the increase with time indicates that [Ca²⁺], increased with the duration of oxygen-glucose deprivation. However, the magnitude of the increase in [Ca²⁺], in layer II/III neurons was significantly greater than those seen in the other layers (P < 0.01). In contrast, there were no significant differences among layers IV–VI (Fig. 3).

Effects of amino acid antagonists on [Ca²⁺], accumulations during oxygen-glucose deprivation

We tested several kinds of amino acid antagonists to elucidate what kind of transmitters and receptors might be responsible for the laminar difference observed in the intracellular Ca²⁺ accumulations. In the presence of kynurenic acid (500 μM), a significant laminar difference was not observed (Fig. 4A). Kynurenic acid reduced the magnitude of the [Ca²⁺], increases previously seen in all layers during oxygen-glucose
deprivation, but its effect was strongest on cells in layer II/III (Fig. 6B). On this basis, the laminar difference in intracellular Ca\(^{2+}\) accumulation appeared to be glutamate-dependent. Bicuculline (10 \(\mu\)M) also reduced the magnitude of the intracellular Ca\(^{2+}\) accumulations in all layers during oxygen-glucose deprivation (Fig. 6D) (see also Fukuda et al. 1998); however, a significantly greater increase in [Ca\(^{2+}\)] was still seen in layer II/III than in the other layers (\(P < 0.05\), Fig. 4B). Thus, although it might have some involvement, the GABA\(_A\) receptor is unlikely to be solely responsible for the laminar difference in the [Ca\(^{2+}\)] increases.

Selective glutamate receptor blockers were used in an attempt to determine which subtype of glutamate receptor might mediate the differential laminar [Ca\(^{2+}\)] increases induced by oxygen-glucose deprivation. CNQX (10 \(\mu\)M) reduced the magnitude of the [Ca\(^{2+}\)] increases in all layers during oxygen-glucose deprivation (Fig. 6C); however, a significantly greater increase in [Ca\(^{2+}\)] was still seen in layer II/III (\(P < 0.01\), Fig. 5A). AP5 (20 \(\mu\)M), like CNQX (although to lesser extent), reduced the magnitude of the [Ca\(^{2+}\)] increases in all layers during oxygen-glucose deprivation (Fig. 6A). Moreover, a laminar difference was no longer observed in the presence of AP5 (Fig. 5B).

**Laminar differences in the protective effects of amino acid antagonists**

As shown in Fig. 6, the percentage inhibition produced by amino acid antagonists of the oxygen-glucose deprivation-induced intracellular Ca\(^{2+}\) accumulations was calculated for each layer. The percentage inhibition produced by AP5 (20 \(\mu\)M) was significantly greater in layer II/III than in the other layers: layer II/III, 78.6 ± 18.0%; layer IV, 62.6 ± 35.3%; layer V, 64.7 ± 35.5%; layer VI, 69.5 ± 27.3% (Fig. 6A). The percentage inhibition produced by kynurenic acid (500...
Recent reports have shown that glial cells in slice preparations also respond to glutamergic agonists (Porter and McCarthy 1995) and to ischemia (Duffy and MacVicar 1996) by showing an [Ca\textsuperscript{2+}] increase. For this reason, discrimination of neurons from glial cells is necessary when recording [Ca\textsuperscript{2+}] in slices. In the present study, we used a 10 objective that enabled us to record from neocortical layers II–VI.

**DISCUSSION**

The results of the present study indicate that extracellular accumulation of glutamate is partly responsible for oxygen-glucose deprivation-induced intracellular Ca\textsuperscript{2+} accumulations in neocortical pyramidals and that layer II/III cells are the most susceptible. The differential susceptibility could be caused by some differences in glutamatergic synaptic transmission mediated by NMDA receptors. Our data also suggest that GABA, as well as glutamate, does play some part in inducing [Ca\textsuperscript{2+}] increases during oxygen-glucose deprivation.

**FIG. 3.** Differential increases in [Ca\textsuperscript{2+}], according to neocortical layer during application of oxygen-glucose deprived standard ACSF (control) at room temperature. Each data point represents mean ± SEM of normalized R\textsubscript{F340/F380} values for identified single pyramidal neurons in each neocortical layer (n = 140 in each layer from a total of 20 independent experiments). The normalized R\textsubscript{F340/F380} increased with time, indicating that [Ca\textsuperscript{2+}] increased with the duration of oxygen-glucose deprivation. The period of oxygen-glucose deprivation is indicated by the bar at the top of the graph. Note that the degree of [Ca\textsuperscript{2+}] accumulation in layer II/III neurons was significantly greater than that in the other layers (*P < 0.01, repeated-measures ANOVA followed by Tukey-Kramer test).

![Normalized Ratio (F340/F380) vs Time (min)](image)

**FIG. 4.** Effects of amino acid receptor antagonists on intracellular Ca\textsuperscript{2+} accumulation in neocortical layers during oxygen-glucose deprivation at room temperature. Graphs are to be compared with Fig. 3. **A**: kynurenic acid (500 μM) reduced the magnitude of the [Ca\textsuperscript{2+}] increases seen in all layers during oxygen-glucose deprivation. A significant difference between layers in terms of their [Ca\textsuperscript{2+}] increase was not observed, indicating that the laminar difference shown in Fig. 3 was glutamate dependent (repeated-measures ANOVA). **B**: Bicuculline (10 μM) tended to reduce the magnitude of the [Ca\textsuperscript{2+}] increases seen in all layers during oxygen-glucose deprivation. However, a significantly greater [Ca\textsuperscript{2+}] increase was still observed in layer II/III (†P < 0.05, repeated-measures ANOVA followed by Tukey-Kramer test). Each data point in graphs A and B represents mean ± SE of normalized R\textsubscript{F340/F380} values for neurons in each layer (n = 140 in each layer from a total of 20 independent experiments).
Although we did not measure extracellular glutamate concentration, it would appear that the extracellular level of glutamate increased in all layers, as antagonists were effective in all layers. The laminar difference in the percentage inhibition of the oxygen-glucose deprivation-induced \([\text{Ca}^{2+}]_i\) increases produced by glutamate receptor antagonists suggests that the composition of the receptor population might differ by layer. In the hippocampus, it has been reported that, while the amount of released glutamate did not differ by region, the changes in \([\text{Ca}^{2+}]_i\) did show regional variations corresponding to the reported differences in the NMDA receptor density (Mitani et al. 1990, 1991). In the present study, AP5 and kynurenic acid were both most effective in layer II/III, whereas CNQX was most effective in layer VI. These results are compatible with a previous autoradiographical report showing that the level of \([\text{Ca}^{2+}]_i\) permeable NMDA receptors is relatively high in layer II/III and relatively low in the deeper layers (Monaghan and Cotman 1985).

The relatively low temperature employed in the present study might have affected the postsynaptic sensitivity of glutamate receptors, and thus generated laminar differences in receptor sensitivity. However, hypothermia does not change the degree of \([\text{Ca}^{2+}]_i\) elevation evoked by kainate or NMDA application (Bruno et al. 1994). In any case, our results are consistent with those of Lin et al. (1994) who reported that the largest \([\text{Ca}^{2+}]_i\) rise was seen in layer II/III of neocortical slices on application of NMDA, in rats at a similar developmental stage as those used in the present study. Thus, NMDA receptors may play a critical role, though other receptors may also play a part, in the tendency of layer II/III cells to experience a greater intracellular \([\text{Ca}^{2+}]_i\) accumulation during oxygen-glucose deprivation, as indicated by the present results.

There are some discrepancies between the results of our study and those of previous studies. In more mature rat neocortex, glutamatergic antagonists were not so effective in protecting against \([\text{Ca}^{2+}]_i\) increases (Bickler and Hansen 1994; Kral et al. 1993). The NMDA receptor antagonist, AP5, had no effect on hypoxia-induced increases in extracellular \([\text{Ca}^{2+}]_i\) concentration in the adult rat neocortex (Kral et al. 1993). However, Kral et al. (1993) applied hypoxic conditions for only a short period, whereas we applied oxygen-glucose deprivation continuously for 45 min. During brief ischemia, changes in the extracellular accumulation of glutamate might be only transient and too small to increase \([\text{Ca}^{2+}]_i\) (Matsumoto et al. 1996; Takata et al. 1995). Indeed, in glucose-deprived hippocampal slices, glutamate accumulation in the extracellular space is biphasic, decreasing rapidly after the reintroduction of glucose and then showing a sustained increase concomitant with a massive \([\text{Ca}^{2+}]_i\) increase (Takata et al. 1995). Furthermore, in an in vitro model in which ischemia is produced for 60 min in cultured cells, the induced \([\text{Ca}^{2+}]_i\) increase was also biphasic: a transient increase was followed by a sustained increase, and the latter was AP5-sensitive (Tymianski et al. 1993). These results are compatible with ours in that the sustained \([\text{Ca}^{2+}]_i\) increases were glutamate antagonist-sensitive. An alternative explanation could be that the immature cortex used in the present study is more susceptible to NMDA overstimulation than the adult brain (Johnston 1995; Young et al. 1991).
FIG. 6. Laminar differences in the effects of excitatory and inhibitory amino acid antagonists on oxygen-glucose deprivation-induced intracellular Ca\textsuperscript{2+} accumulation. Both excitatory and inhibitory amino acid antagonists reduced the magnitude of the [Ca\textsuperscript{2+}] increase seen in all neocortical layers during oxygen-glucose deprivation. The % inhibition of the oxygen-glucose deprivation-induced [Ca\textsuperscript{2+}] elevation was calculated for each layer at 50 min after the beginning of the recording period (45 min after the initiation of oxygen-glucose deprivation). This was done by taking the difference between an individual normalized R(340/F380) value with antagonist and the mean normalized R(340/F380) value without antagonist (control) and dividing by the latter (X100%). § P < 0.0001, $ P < 0.002; indicates significance of difference from control (i.e., 0% inhibition). Mann-Whitney U-test. A: the % inhibition induced by AP5 (20 µM) was significantly greater in layer II/III than in the other layers (n = 119 in each layer). B: the % inhibition induced by kynurenic acid (500 µM) was also significantly greater in layer II/III (n = 140 in each layer). C: the % inhibition induced by CNQX (10 µM) was not different from the inhibitions in the other layers, whereas in layer VI it was significantly greater than those in layers IV and V (n = 105 in each layer). D: the effect of bicuculline (10 µM) did not differ significantly between layers, although it reduced the magnitude of the [Ca\textsuperscript{2+}], increases from control in all layers (n = 140 in each layer). Values shown are mean ± SE. * P < 0.01, † P < 0.05, one-way ANOVA followed by Tukey-Kramer test.

Indeed, Bickler et al. (1993) reported that the NMDA receptor antagonist, MK-801, delayed the hypoxia-induced [Ca\textsuperscript{2+}] increase in the neocortex of rats younger than P14, but had no such effect in rats older than 2 wk.

The latencies we measured to the abrupt [Ca\textsuperscript{2+}] increases were longer than those reported previously (Bickler et al. 1993; Bickler and Hansen 1994) and also longer than those recorded for the anoxic depression of the extracellular DC potential and the concomitant decrease in extracellular Ca\textsuperscript{2+} concentration (Kral et al. 1993). However, those authors performed experiments at 34–37°C using more mature rats, whereas we used immature rats at 22–24°C. Hypothermia reduces glutamate release (Bruno et al. 1994) and the latency of the [Ca\textsuperscript{2+}] rise is much longer in younger animals (Bickler et al. 1993; Friedman and Haddad 1993). In addition, within our observation period, there was no clear evidence of the death of neurons (which would have indicated sustained [Ca\textsuperscript{2+}], increases). This would probably be due to our use of hypothermia, which works as a neuroprotective in cortical slices and in cortical cultures exposed to hypoxic or ischemic conditions (Bruno et al. 1994; Hiramatsu et al. 1993).

Extracellular accumulation of GABA as well as glutamate occurs during ischemia in the cortex (Matsumoto et al. 1996; O’Regan et al. 1995). The results of the present study indicate that GABA acts to elevate [Ca\textsuperscript{2+}], since bicuculline reduced the [Ca\textsuperscript{2+}] increase seen during oxygen-glucose deprivation. It has been suggested that the GABA reversal potential shifts to more positive levels during hypoxia/ischemia in slices (Katchman et al. 1994; Khazipov et al. 1995; Luhmann et al. 1993). Therefore a GABA\textsubscript{A}-mediated response could be reversed to depolarization by a positive shift in the GABA reversal potential over the resting potential.

Large and prolonged increases in [Ca\textsuperscript{2+}], like those observed in the present study, may mediate various changes that accentuate cell excitability, including inhibition of the Na\textsuperscript{+}-K\textsuperscript{+} pump (Fukuda and Prince 1992b), decreases in GABA responses (Inoue et al. 1986; Stelzer et al. 1988), and enhancement of glutamate (Coulter et al. 1992) and NMDA (Markram and Segal 1991) responses. In addition, several enzyme families which require Ca\textsuperscript{2+} for their activation, such as NO synthase, which is most abundant in layer II/III (Vaischianoff et al. 1993), will be activated (Choi 1990). Furthermore, for a given elevation in [Ca\textsuperscript{2+}], the cytotoxicity induced by Ca\textsuperscript{2+} overload is more severe when this is induced by NMDA agonists than by non-NMDA agonists (Tymianski et al. 1993). Although we did not measure absolute values for [Ca\textsuperscript{2+}], Bickler et al. (1993) provided...
evidence that it was elevated to over 800 nM by anoxia in the neocortex from P8–14 rats. Thus, the sustained increase in \([\text{Ca}^{2+}]_i\) in the present study could indicate \(\text{Ca}^{2+}\) overload, which would lead to a subsequent deterioration. Therefore, the NMDA receptor-mediated differential susceptibility of layer II/III pyramidal neurons to an oxygen-glucose deprivation-induced \([\text{Ca}^{2+}]_i\), increase may imply a particular vulnerability of this layer to ischemia-induced excitotoxicity. Indeed, Freund et al. (1990) reported that layer II/III pyramidal neurons are one of the populations most vulnerable to ischemia. Since the developmental stage of the rat cerebral cortex studied in the present report corresponds approximately to that of the newborn human infant (Romijn et al. 1991), such a differential laminar vulnerability might play an important role in determining both the topographical characteristics of neurotoxicity in the perinatal period and its sequelae later in life.

We thank Dr. R. Timms for language-editing this manuscript. This work was supported by Grants-in-Aid 07680897, 09260226, and 09680817 to A. Fukuda from the Ministry of Education, Science, Sports and Culture, Japan.

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Received 27 February 1997; accepted in final form 10 September 1997.

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Mitani, A., Kadoya, F., Nakamura, Y., and Katoaka, K. Visualization


