Dual Effect of Glutamate on GABAergic Interneuron Survival during Cerebral Cortex Development in Mice Neonates

In term and preterm neonates, massive glutamate release can lead to excitotoxic white-matter and cortical lesions. Because of its high permeability toward calcium, the N-methyl-D-aspartic acid (NMDA) receptor is thought to play an important role in excitotoxic lesions and NMDA antagonists therefore hold promise for neuroprotection. We found that, in neonatal mouse cortex, a given NMDA concentration exerted either excitotoxic or antiapoptotic effects depending on the cortical layers. In layer VI, NMDA led to excitotoxicity, sustained calcium mobilization, and necrosis of Gad67GFP neurons. In the immature layers II–IV, NMDA decreased apoptosis and induced transient calcium mobilization. The NMDA antagonist MK801 acted as a potent caspase-3 activator in immature layers II–IV and affected gamma aminobutyric acid (GABA)ergic interneurons. The apoptotic effect of MK801-induced BAX expression, mitochondrial potential collapse and caspase-9 activation. In vivo BAX small interfering ribonucleic acid and a caspase-9 inhibitor abrogated MK801-induced apoptosis and pyknotic nucleus formation. Ketamine, an anesthetic with NMDA antagonist properties, mimicked the apoptotic effects of MK801. These data indicate a dual effect of glutamate on survival of immature and mature GABAergic neurons and suggest that ketamine may induce apoptosis of immature GABAergic neurons.

Keywords: bax, development, GABA, ketamine, MK801

Introduction

Hypoxia-ischemia is among the main causes of brain damage in preterm and full-term neonates (Alvarez-Diaz et al. 2007). At a clinical level, the brain lesions may result in cerebral palsy and mental retardation (Volpe 2009). Cell types are differently affected by hypoxia-ischemia. For example, maturation-dependant vulnerability has been described for oligodendrocytes and white matter lesions occur preferentially in preterm infants (Back et al. 2001, 2007) whereas neurons in the cortical layers and basal ganglia are predominantly affected in full-term infants (Jensen 2002). Cell death due to hypoxia-ischemia involves several mechanisms including adenosine triphosphate depletion (Garnier et al. 2002), neurotrophin level reduction (Riikonen et al. 1999), generation of reactive oxygen species (Lievre et al. 2001) and inflammation (Aly et al. 2006). Among them, the massive release of glutamate represents a major process (Johnston 2005; Laudenbach et al. 2001). Overstimulation of the ionotropic glutamate receptors triggers a rapid inflow of sodium and calcium, leading to excitotoxic damage (Follett et al. 2004). Although glutamate-induced excitotoxicity involves several receptor types (Hilton et al. 2006), the NMDA receptor is thought to play a crucial role based on its high permeability to calcium (Mishra et al. 2001). Consistent with this hypothesis, several reports indicate that N-methyl-D-aspartic acid (NMDA) agonists are powerful inducers of neuronal cell death and suggest that NMDA antagonists may exert a protective effect (Marret et al. 1996; Schulz et al. 1998; Arias et al. 1999).

Despite evidences in favor of a pivotal role of glutamate in the development of white matter and/or cortical lesions (Rangon et al. 2007; Khwaja and Volpe 2008), the ability of NMDA antagonists to afford neuroprotection of the immature brain is increasingly debated (Ikonomidou et al. 2000; Wang et al. 2006). Indeed, the expression profile of NMDA receptor subunits changes during neurodevelopment (Zhou and Baudry 2006) and the kinetics of excitatory postsynaptic currents vary across receptor oligomers (Barth and Malenka 2001). The specific characteristics of the receptor subunit arrangements in the immature brain may influence the effect of glutamate on cell death (Liu et al. 2007). Ketamine, an analgesic and anaesthetic drug used in pediatric intensive care (Bhutta 2007), acts as a non competitive antagonist of the NMDA receptor (Orser et al. 1997). Several reports described neuroprotective effects of ketamine after brain injury (Shapira et al. 1994; Proescholdt et al. 2001). However, these studies were performed in adults. Although deleterious effects of ketamine have been reported in young animals (Ikonomidou et al. 1999), these studies were conducted after neuronal migration was completed and the underlying molecular mechanisms were not elucidated.

The aims of the present study were to characterize 1) the apoptotic and necrotic effects of NMDA and its antagonist MK801 on cortical slices from mice neonates, 2) the phenotype and functional capabilities of cell types affected by NMDA and MK801, 3) the molecular pathway involved in MK801-induced apoptosis, and 4) the effects of ketamine on apoptosis in the neocortex of mice neonates.

Materials and Methods

Animals

NMRI (National Marine Research Institute) mice purchased from Janvier (Le Genest Saint-Ise, France) were kept in a temperature-controlled room (21 ± 1°C) with a 12-h/12-h light/dark cycle (lights on from 7 AM to 7 PM) and free access to food and tap water. FVB-Tg(GadGFP)5704Swn transgenic mice (# 003718) were obtained from The Jackson Laboratory (Bar Harbor, ME). In these transgenic mice, gamma aminobutyric acid (GABA)ergic interneurons arising from the ganglionic eminences express enhanced green fluorescent protein under the control of the mouse Gad1 gene promoter (Oliva et al., 2000). Animal care and manipulations complied with recommendations.
issued by the French and European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC, license n°. 21CAAB35) and under the supervision of authorized investigators (B.J.G., authorization n°. 7687 from the Ministère de l’Agriculture et de la Pêche).

**Chemicals**

NMDA, the AMPA/Kainate receptor antagonist CNQX, and the NMDA receptor antagonist MK801 were purchased from Tocris (Bristol, UK). 7-Aminoactinomycin D (7-AAD), Hoechst 33258, glutamate, β-nicotinamide adenine dinucleotide (NADH), and sodium pyruvate were from Sigma Aldrich (Saint-Quentin Fallavier, France). The ratiometric intracellular calcium probe Fura-2, the pluronie F-127, and the CellTracker Red were from Invitrogen (Cergy Pontoise, France). The Apo-ONE homogeneous caspase-3/7 kit, the caspaseGlo-8 and -9 kit, the caspaseGlo-8 or caspase-Glo-9 (Z-LEHD-FMK) inhibitors were purchased by R&D systems Europe (Abingdon, UK). The rabbit polyclonal antibody against cleaved caspase-3 (#9661) was purchased by Cell Signaling Technology (Boston, MA). The rabbit polyclonal antibody against gamma-aminobutyric acid (GABA; #A2052) was from Sigma. The rabbit polyclonal antibody against BAX and the goat polyclonal antibodies directed against glial fibrillary acidic protein (GFAP; #sc-6170), MAP-2 (#sc-5559), and Olig-2 (#sc-19969) were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 donkey anti-rabbit IgG (A-21206) and Alexa Fluor 594 donkey anti-goat IgG (A-21042) were from Invitrogen. ECL RPN 2108 kit for western blot experiments was from Amersham (Orsay, France). In vivo ready Bax small interfering ribonucleic acid (siRNA) (siBax) were designed by Ambion-Applied Biosystems.

**Preparation and Treatment of Cerebral Slices from Postnatal Day 2 Mice**

Cortex brain slices were obtained from postnatal day 2 (P2) mice because several previous studies demonstrated that excitotoxic cortical lesions observed at this developmental stage presented similarities with those observed in preterm infants (Mespes et al. 2005). The mice were sacrificed by decapitation and their brains rapidly dissected in order to isolate the hemispheres. The overlying meninges were carefully removed and the neocortex immediately placed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 125; KCl, 3; CaCl2, 2; NaH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 26; D-glucose 10; pH 7.4. Transverse slices (250 μm) were cut at 4 °C by using a vibratome VT1000S Leica (Rueil-Malmaison, France) then transferred into 24-well Costar plates (Cambridge, MA) containing aCSF and incubated for a 30-min recovery period at 37 °C in a humidified incubator under a controlled atmosphere of 5% CO2/95% air. Then, the slices were washed with fresh aCSF and treated for 10 min to 6 h, at 37 °C, with 100 μM glutamate, (12.5-800 μM) NMDA, 20 μM MK801, 20 μM CNQX, 10 μM of the caspase-8 inhibitor (IETD-FMK), and/or 20 μM of the caspase-9 inhibitor (Z-LEHD-FMK).

**Lactate Dehydrogenase Activity Assay**

Lactate dehydrogenase (LDH) activity in the extracellular medium was used as an index of cell lysis representative of necrosis (Cechetti et al. 2007). After exposure of brain slices to aCSF alone or with glutamate agonists and/or antagonists for 6 h, 30 μL of aCSF were collected and mixed with 100 μL of a freshly made solution containing 0.5 mM NADH. Conversion of NADH to its oxidize form NAD+ was initiated by adding 20 μL of 5 mM sodium pyruvate. LDH activity was then monitored for 15 min by measuring the decrease of NADH absorbance using spectrophotometry at 340 nm with a Chameleon plate reader (M LISTOKa, Turku, Finland).

**Visualization of Cell Death**

Cell death associated with plasma membrane permeability was visualized after 6 h of treatment using the DNA intercalator 7-AAD, which produces red fluorescence in the nuclei of dead cells. Briefly, brain slices were washed with phosphate-buffer saline (PBS) at 37 °C and incubated for 5 min with 15 μM 7-AAD. Slices were then washed 3 times for 5 min in PBS at 37 °C and fixed overnight at 4 °C with 4% paraformaldehyde (PFA) in PBS prior to immunohistochemical studies. Dead cells were visualized using a fluorescence videomicroscope system DMI 6000 B Leica (Rueil-Malmaison, France) at 530-nm excitation and 585 nm emission wavelengths.

**Intracellular Calcium Measurements**

Transverse slices were incubated for 15 min in aCSF containing 10 μM Fura-2 AM and 0.03% pluronie F-127. The slices were then washed twice for 5 min in fresh aCSF, and each slice was transferred in a 35-mm dish containing 1 mL of aCSF and immobilized using a nylon mesh. The temperature of the aCSF was kept constant at 37 °C and the slice was rinsed with aCSF. Two-fold concentrated solutions of NMDA, MK801, and ketamine were perfused in an equal volume of aCSF baring the slice in order to reach final concentrations similar to those used for measurement of LDH and caspase-3 activities. The fluorescent signals associated with calcium-free and calcium-bound Fura-2 were measured by alternatively exciting the slices at 340 and 380 nm using a fluorescence Leica DM microscope equipped with a rapid shutter wheel. The emitted fluorescence was collected at 510 nm and a ratio of both signals was calculated by the Metamorph software (Roper Scientific, Eivy, France). The data were exported to the biostatistic software Prism (GraphPad, Inc., San Diego, CA), which was used to compute the maximal fluorescence intensity (MI) and the area under the curve (AUC).

**Morphometric Analysis of NMDA-Induced Necrosis on GadGFP-Positive Cells**

Transverse slices from P2 GadGFP transgenic mice were cultured under a fluorescent Leica DM microscope and rinsed with fresh aCSF. GadGFP-positive cells present in layer VI were exposed to NMDA (intrapiette concentration 800 μM) in absence or presence of MK801 (40 μM intrapiette) and Z-series images of GadGFP-positive cells were acquired every 10 min and saved in TIFF format using a computer-assisted image analysis station Metamorph (Roper Scientific). Then, regions of interest (ROIs) corresponding to the GadGFP-positive cell bodies were selected and their areas were quantified and integrated to compute cell volume using the Mercator Software (Exploра Nova, La Rochelle, France). The death of GadGFP-positive cells after exposure to NMDA was visualized by videomicroscopy using 7-AAD. A first 7-AAD labeling was done at the beginning of the video recording (t = 0). Then, GadGFP-positive cells were exposed for 50 min to aCSF alone, NMDA (800 μM; intrapiette concentration) and/or MK801 (40 μM intrapiette). Finally, a second 7-AAD labeling was done at the end of the video recording (t = 50 min). The comparison of the overlays "GadGFP/7-AAD" acquired at t = 0 and t = 50 min was used to visualize GadGFP-positive cells died after exposure to NMDA.

**Caspase Activity Assays**

For caspase-3 activity measurements, cortices from brain slices were resuspended in 500 μL of hypotonic lysis buffer and 20 μg of proteins were incubated at 30 °C with 100 μL of caspase-3 buffer containing 1 μL of the caspase-3 substrate Z-DEVD-R110 provided within the Apo-ONE homogeneous caspase-3/7 kit (Promega, Fitchburg, WI). Fluorescence intensity was quantified every 5 min for 2 h at excitation and emission wavelengths of 485 and 520 nm, respectively, using a Chameleon plate reader. For caspase-8 and caspase-9 activity measurements, 100 μL of Caspase-Glo 8 or Caspase-Glo 9 Reagents were added in a white-walled 96-well plate containing 100 μL of blank or treated cell extracts. The plate was gently mixed and luminescence was read using a Chameleon plate reader at room temperature for 30 min to 3 h.

**Intracellular Labeling of Caspase Activity**

Caspase activity was detected and visualized using the substrate CaspACE fluoroisothiocyanate (FITC)-VAD-FMK in situ marker Promega (Charbonnieres les Bains, France). This fluorescent caspase
inhibitor penetrates within the cell, where it binds to activated caspasess, thereby serving as an in situ marker of apoptosis. Briefly, slices were incubated with 10 μM CaspACE FITC-VAD-FMK for 20 min at 37 °C, and then washed 3 times with PBS. Green fluorescence was visualized at the excitation and emission wavelengths of 485 and 520 nm, respectively. CaspACE FITC-VAD-FMK fluorescent probe was used on fresh brain slices to enable the identification and dissection of cortices prior to protein extraction and Bax western blotting. Some of the slices exposed to CaspACE FITC-VAD-FMK were fixed with 4% PFA in PBS overnight and then used for immunohistochemical studies.

**Immunohistochemistry**

Brain slices previously fixed overnight with 4% PFA in PBS were incubated overnight at 4 °C with various primary antibodies diluted in incubation buffer (PBS containing 1% bovine serum albumin [BSA] and 3% Triton X-100). Then, the slices were rinsed twice with PBS for 20 min and incubated with the same incubation buffer containing the adequate secondary antibody, that is, Alexa Fluor 488 donkey antirabbit IgG and Alexa Fluor 594 donkey anti-goat IgG. Cell nuclei were visualized by incubating the slices for 5 min with 1 μg/mL Hoechst 33258 in PBS. Fluorescent signals were observed with a Leica DMI 6000B microscope. The specificity of the immunoreactions was controlled by substituting the primary antibodies by PBS.

**Visualization and Quantification of Mitochondrial Integrity**

Mitochondrial membrane potential was assessed using the ratiometric probe JC-1 from Molecular Probes (Leiden, the Netherlands). In healthy cells, the intact membrane potential allows the lipophilic dye JC-1 to enter into the mitochondria where it accumulates and aggregates, producing an intense orange signal. In cells whose mitochondria have low membrane potential, the JC-1 remains monomeric and stays in the cytoplasm, where it emits green fluorescence (Smiley et al. 1991). Acute brain slices were treated for 6 h at 37 °C with 400 μM NMDA or 20 μM MK801, incubated for 30 min with 3 μL/mL JC-1, and finally washed twice for 5 min with PBS at the same temperature. Fluorescence was visualized immediately without prior fixation at the 485-nm excitation and 530-nm (green) emission wavelengths and at 550-nm excitation and 590 nm (orange) emission wavelengths. The green and orange signals were acquired and saved in TIFF format using a computer-assisted image analysis station Metamorph. The fluorescence intensity profiles corresponding to monomeric and aggregated JC-1 were used to quantify the 530/590 ratio in the ROIs. For each fluorescent signal, a background level was defined in a negative region of the images.

**Western Blot Analysis**

Acute slices were incubated for 3 h with 400 μM NMDA or 20 μM MK801. Total cellular proteins from 2 slices were extracted using 900 μL of the lysis buffer containing 1% Triton X-100, 50 mM Tris/His, and 10 mM ethylenediamine tetraacetic acid. The homogenate was centrifuged (20,000 × g, 4 °C, 15 min) and the supernatant proteins were precipitated by addition of ice-cold 10% trichloroacetic acid. The extract was centrifuged (15,000 × g, 4 °C, 15 min) and washed twice with alcohol/ether (70/30 v/v). The pellet was denatured at 100 °C for 5 min in 50 μL of Tris/His (pH 7.5) containing 20% glycerol, 0.7 M 2-mercaptoethanol, 0.004% (w/v) bromophenol blue and 3% (w/v) sodium dodecyl sulfate (SDS) then electrophoresed on 10% SDS-polyacrylamide gel. After separation, the proteins were electrically transferred onto a polyvinylidene difluoride membrane (PerkinElmer Life Sciences, Boston, MA). The membrane was incubated with blocking solution (1% BSA in Tris-buffered saline containing 0.05% Tween 20) at room temperature for 1 h and revealed with antibodies against Bax and actin (Santa Cruz Biotechnology, Santa Cruz, CA) using an ECL RPN 2108 chemiluminescence detection kit (PerkinElmer Life Sciences, Boston, MA). Autoradiographic films were quantified using an image analysis system (Samba, Grenoble, France). Commercial markers (see blue pre-stained standard) were used as molecular weight standards.

**In vivo Ready Bax siRNA injections**

Silencer In vivo Ready Bax siRNAs were designed against the target gene Bax (Ambion, Applied Biosystems, Foster City, CA). Sequences of the oligonucleotides used were: sense 5′ → 3′, GGCCCUUGGGACUAAAGUGUtt and antisense 5′ → 3′, CACUUAUGUGGCAGGGCCti. Fifty micrograms of siBax were diluted in 2.5 mL of an injection solution consisting in 2% dimethyl sulfoxide and 0.9% NaCl. The same injection solution without siRNA was given to the sham mice, whereas the control mice received no injection. Newborn mice (postnatal day 0; P0) were anesthetized by isoflurane inhalation then injected in the frontal cortex of the right hemisphere. Pilot injection tests were done using CellTracker Red to standardize the injection protocol. A second injection was given on P1, 24 h after the first injection. The animals were killed by decapitation on P2, the brains were rapidly removed and cortical slices were obtained and cultured ex vivo for 6 h with or without MK801. The slices were then used for immunohistochemical and apoptosis studies.

**Visualization and Quantification of Pyknotic Nuclei**

Cortical slices from control and siBax-injected animals were incubated for 6 h in ACSF alone or with 20 μM MK801. Five minutes before the end of the treatment, slices were treated for 5 min with 1 μg/mL Hoechst 33258, washed twice with PBS for 5 min and fixed overnight with 4% PFA. TIFF format images of the Hoechst 33258 positive nuclei were acquired, and the proportion of pyknotic nuclei in the ROIs was quantified by segmentation using the Metamorph image analysis station (Roper Scientific). Data were expressed as the ratio of pyknotic nuclei over the total number of nuclei.

**Statistical Analysis**

Statistical tests were run using the biostatistic software Prism (GraphPad Inc., San Diego, CA). Reported data are mean ± SEM of at least 3 independent experiments performed at least in triplicate. The 1-way ANOVA followed by Tukey’s post hoc test was used to compare the effects of treatments on LDH and caspase-3 activities as well as on cell volumes. The Mann-Whitney test was used to analyze the AUCs and the MFIs obtained by calcimetry. Cell populations with different intracellular calcium profiles were compared using the Chi-square test.

**Results**

**Effects of Glutamate, NMDA, MK801, and CNQX on Necrotic Cell Death**

Exposure of P2 cerebral slices to glutamate (400 μM) for 6 h induced a significant increase in LDH activity, a parameter traducing a cell lysis and representative of necrosis (P < 0.01; Fig. 1A). When compared with control, the NMDA antagonist MK801 (20 μM; P < 0.01) as well as the AMPA/kainate antagonist CNQX (20 μM; P < 0.05) administered alone reduced the LDH activity, suggesting a basal excitotoxic process in control slices (Fig. 1A). However, MK801 (20 μM) was more potent than CNQX (20 μM) for preventing glutamate-induced cell death (Fig. 1A). No additive effect was quantified when the 2 antagonists were coincubated (Fig. 1A). Treatment of P2 cerebral slices with NMDA (400 μM) increased LDH activity by 28.3 ± 4.6% (P < 0.01; Fig. 1B). The cytotoxic effect of NMDA was markedly decreased by MK801 (20 μM; P < 0.001), whereas CNQX (20 μM) had no significant effect (Fig. 1B). When the 2 antagonists were used together, CNQX did not modify the protective effect of MK801 against NMDA-induced cell death (Fig. 1B). To correlate LDH activities with histological findings and cell death, we examined P2 cerebral slices stained with 7-AAD (Fig. 1C). At P2, development of the cortical layers is not totally achieved and although layers VI and
Glutamate (400 μM) - + - + + -
MK801 (20 μM) - + - + + -
CNQX (20 μM) - + - + + -

GLUTAMATE ACTIVITY (SLOPE COEFFICIENT)

Figure 1. Effects of glutamate, NMDA, MK801, and CNQX on LDH activity and cell death. (A) Quantification of LDH activity in cortical slices from 2-day-old mice neonates incubated during 6 h in aCSF alone (control) or exposed to glutamate (400 μM) in the absence or presence of the antagonists MK801 (20 μM) and CNQX (20 μM). One-way ANOVA test showed a group effect (F = 17.15; P < 0.0001) and the Tukey’s multiple comparison test indicated **P < 0.01 versus control and ***P < 0.01 versus glutamate. (B) Quantification of LDH activity in cortical slices incubated during 6 h in aCSF alone or exposed to NMDA (400 μM) in the absence or presence of the antagonists MK801 (20 μM) and the AMPA/kainate antagonist CNQX (20 μM) used alone significantly increased caspase-3 activity (P < 0.01; Fig. 1A). When glutamate and MK801 were coincubated, MK801 markedly blunted the inhibitory effect of glutamate on caspase-3 activity (P < 0.001; Fig. 1A). Surprisingly, CNQX (20 μM) modified neither the effect of glutamate on caspase-3 activity nor the action of MK801 coincubated with glutamate (Fig. 1A). Treatment of cerebral slices with NMDA (400 μM) mimicked the inhibitory effect of glutamate on caspase-3 activity (Fig. 2B). MK801 (20 μM) alone increased the activity of caspase-3 by 24.9 ± 2.4% (P < 0.01) and abolished the effect of NMDA (Fig. 2B). In contrast, although CNQX (20 μM) alone significantly increased the caspase-3 activity, it did not affect the inhibitory effect of NMDA on the protease (Fig. 2B). Dose-effect studies revealed that NMDA respectively increased and reduced LDH and caspase-3 activities in a dose-dependent manner (ED50 = 313 ± 53 μM for LDH and ED50 = 10 ± 1 μM for caspase-3; Fig. 2C). For concentrations ranging from 200 to 800 μM, a given dose of NMDA induced significant and opposite effects on both activities (Fig. 2C). The visualization of cortical structures expressing cleaved (potentially activated)-caspase-3 was performed by immunohistochemistry (Fig. 2D). In control slices, immunoreactivity was located mainly in the developing layers II to IV (Fig. 2D, left). NMDA (400 μM) decreased cleaved caspase-3 immunolabeling in these layers (Fig. 2D, middle), whereas MK801 (20 μM) alone (data not shown) or coincubated with NMDA (Fig. 2D, right) markedly increased cleaved caspase-3 immunoreactivity in the immature layers II–IV and the highest part of layer V. The overlay of the 7-AAD signal (Fig. 1C) and the cleaved caspase-3 immunoreactivity acquired simultaneously on the same slice, clearly revealed different distribution patterns indicating that a same concentration of NMDA exerted dual effects in the immature cortex: 1) inhibition of caspase-3 cleavage in immature layers II–IV and 2) excitotoxic cell death in the deeper layers V and VI (Fig. 2E, middle). NMDA receptor blockade with MK801 (20 μM) induced the opposite effect, causing massive induction of cleaved caspase-3 immunoreactivity in the immature layers II–IV (Fig. 2E, right). To further confirm the presence of apoptotic cells in these layers, we performed double labeling with Hoechst and cleaved caspase-3 antibody. At high magnification in the control condition, numerous nuclei exhibited a normal appearance (Fig. 2F, left, arrows) and these cells were caspase-3 negative (Fig. 2F, right, arrows). In contrast, with MK801, many pyknotic and/or fragmented nuclei were seen in the immature layers II–IV (Fig. 2G, left, arrow heads) and overlapped with the cleaved caspase-3 immunoreactivity (Fig. 2G, right, arrow heads).
Effects of Glutamate on Cytosolic Calcium Levels in Organotypic Slices

Based on previous studies which reported that 1) the distribution profiles of NMDA receptor subunits change during brain development (Zhou and Baudry 2006) and 2) NMDA receptor subunits can have differential roles in mediating cell death in vitro (Liu et al. 2007), we measured the ability of NMDA to regulate calcium mobilization in the deep layer VI and in the developing layers II--IV of P2 cerebral slices. After the slices were loaded with the calcium probe Fura-2, numerous fluorescent cells were seen in the corpus callosum and layer VI (Fig. 3A). Ratiometric quantification of basal calcium mobilization in individual cells showed small and spontaneous increases of cytosolic calcium levels (Fig. 3B). Adding NMDA (800 μM intrapipette) in the perfusion bath markedly increased the fluorescence intensity, indicating a strong calcium mobilization into the cells (Fig. 3B,C). The fluorescent signal remained high and sustained even 4 min after...
NMDA was washed away using fresh aCSF (Fig. 3B). In particular, NMDA induced a 8.5 ± 0.7-fold MFI increase and a 24.4 ± 2.4-fold AUC increase (Fig. 3D). Similar experiments were performed on the cortical slices at the level of the immature layers II–IV (Fig. 4A). Under basal condition, no spontaneous calcium flow was recorded in the ROI (Fig. 4B). Adding NMDA (800 μM intrapipette) to the perfusion bath induced a strong increase of intracellular calcium mobilization (Fig. 4B). Contrasting with the recordings performed in the layer VI, the intracellular calcium levels in the immature layers II–IV rapidly decreased without however returning to basal values (Fig. 4B). MK801 (40 μM intrapipette) significantly inhibited the effect of NMDA in both layer VI and the immature layers II–IV (data not shown). Chi-square analysis showed that sustained and transient calcium mobilization after NMDA (800 μM intrapipette) exposure characterized 2 statistically different cell populations ($P < 0.0001$; Fig. 4D). These 2 populations correlated with 2 regional populations, with sustained mobilization in layer VI and transient mobilization in layers II–IV.

**Effect of NMDA on Necrosis of GadGFP-Positive Cells in Layers V–VI**

Because a clinical study suggested a substantial loss of GABAergic neurons in brains from human neonates with hypoxic perinatal brain injuries (Robinson et al. 2006), we investigated the effect of NMDA on survival of GadGFP-positive cells using FVB-Tg(GadGFP)45704Swn transgenic mice. At P2, several GadGFP-positive cells were found in the deep layers V–VI of the neocortex (Fig. 5A). Time-laps recordings acquired as Z-series showed that NMDA (800 μM intrapipette) changed the shape of GadGFP-positive neurons (Fig. 5B). Indeed, cells which initially presented small processes (Fig. 5B, top) gradually became round after NMDA exposure (Fig. 5B, bottom). These morphological changes were associated with a significant increase in GadGFP cell volume ($P < 0.01$; Fig. 5C) and preceded a sudden collapse of the GFP fluorescent signal (Fig. 5D,E). When it was coincubated with NMDA, MK801 prevented the volume increase ($P < 0.01$; Fig. 5C). In addition, 7-AAD labeling performed at the beginning and at the end of the video recording indicated that exposure of brain slices to aCSF had a slight impact on GadGFP cell death (Fig. 5F; arrows). In contrast, treatment of brain slices with NMDA induced a marked increased of cell death and the GadGFP/7-AAD overlay indicated that several of these dead cells were GABAergic (Fig. 5G; arrows).

**Characterization of Cell Types Rescued by NMDA in Layers II–IV**

Previous studies have established that high NMDA concentrations can induce excitotoxic cell death in the white matter
and in the cortical layer VI of neonatal mice (Tahraoui et al. 2001). However, our present data suggest that a same concentration of NMDA could exert both an excitotoxic effect in the cortical layer VI and an antiapoptotic effect on the immature layers II–IV. Taking in consideration these results, we characterized the cell types on which MK801 exerted pro-apoptotic effect in the immature layers II–IV. Double immunolabeling experiments revealed that, among the numerous cleaved caspase-3 positive cells in the immature layers II–IV after MK801 (20 μM) exposure, none were immunoreactive for GFAP, a protein used as a marker of astrocytes (Fig. 6A,B). Similarly, very few cells were immunoreactive for Olig-2, a transcription factor used as a marker for the oligodendrocyte lineage (Fig. 6C,D). In contrast, most of the cleaved caspase-3-positive cells were immunoreactive for MAP2, a microtubule-associated protein used as a neuronal marker (Fig. 6E,F). Triple labeling experiments using a GABA antibody, Hoechst and the fluorescent caspase substrate FITC-VAD-FMK indicated that MK801-induced apoptosis affected the GABAergic interneurons (Fig. 7A–F). Interestingly, several of these apoptotic neurons were characterized by a tangential orientation and they were particularly concentrated in a thin band edging the immature layers II–IV (Fig. 7A,B).

**Apoptotic Cell Death of GABAergic Interneurons in the Developing Neocortex of Mice Neonates**

Caspase-3 is an executioner protease involved in different apoptotic cascades (Siegel 2006). To identify the pathway(s) associated with the pro-apoptotic effect of MK801, we quantified caspase-8 and -9 activities, 2 initiator caspases representative of the extrinsic and intrinsic pathways, respectively. Exposure of neonate brain slices to MK801 (20 μM) for 3 h had no effect on caspase-8 and -9 activities (Fig. 8A). In contrast, MK801 significantly stimulated caspase-9 activity after 6 h (34.2% ± 7.6; P < 0.01; Fig. 8A). Exposure of the slices to the caspase-9 inhibitor Z-LEHD-FMK (20 μM) or to the caspase-8 inhibitor

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**Figure 4.** Effect of NMDA on intracellular calcium mobilization in the immature superficial layers II-IV. (A) Visualization, at low and high magnifications, of nervous cells present in the cortical layers II–IV and labeled with the ratiometric calcium probe Fura-2. (B) Typical recording (upper panel) obtained from a single cell in control condition. Note the absence of small and regular spikes of calcium. Mean recording (lower panel) obtained after exposure of the cortical slices to NMDA (800 μM intrapipette [Int-P]). (C) Statistical analysis of the MFI and the AUC between cells in control condition and exposed to NMDA (800 μM intrapipette). Each value represents the mean (±SEM) of at least 20 cells from 3 independent experiments. ***P < 0.001 versus control with the Mann-Whitney test. (D) Chi-square test analysis comparing the distribution of the intracellular calcium amplitudes in cells from the superficial layers II–IV and from the deep layer VI. Four distributions have been compared: the control condition in layer VI (a), the NMDA (800 μM intrapipette) condition in layer VI (b), the control condition in layer II–IV (c) and the NMDA (800 μM intrapipette) condition in layer II–IV (d).
inhibitor IETD-FMK (10 μM) alone for 6 h induced a significant reduction of the caspase-3 activity compared with the control condition (Fig. 8B). Nevertheless, only Z-LEHD-FMK abrogated the stimulatory effect of MK801 on caspase-3 (Fig. 8B). Immunohistochemical studies using a cleaved caspase-3 antibody showed that the inhibitory effect of Z-LEHD-FMK on caspase-3 activity was essentially localized in the immature layers II–IV (Fig. 8C–E). Caspase-9 activation being associated with induction of the mitochondrial apoptotic pathway, we visualized and quantified the mitochondrial integrity in the immature layers II–IV by using the ratiometric probe JC-1. At low magnification, visualization of the aggregated form of JC-1 (590 nm) revealed that NMDA exposure for 6-h preserved mitochondrial integrity in layers II–IV compared with control (Fig. 9A,B). As with cleaved caspase-3 immunoreactivity, the effect of NMDA on mitochondria was particularly obvious in a subpopulation of cells present

Figure 5. Effect of NMDA on GadGFP-positive cells in the deep cortical layers V–VI. (A) Visualization of GadGFP-positive cells in the immature neocortex at P2. (B) High magnification images showing the effect of NMDA (400 μM) on the morphology of GadGFP-positive neurons (arrows) in the deep cortical layers V–VI. (C) Quantification of the volume of GadGFP-positive cells after exposure to NMDA (800 μM Int-P) in absence or presence of MK801 (40 μM Int-P). One-way ANOVA test showed a group effect ($F = 7.948; P = 0.0004$) and the Tukey’s multiple comparison test indicated **$P < 0.01$ versus control and ###$P < 0.01$ versus NMDA. Each value represents the mean (±SEM) of at least 11 cells from 3 independent experiments. (D, E) Time-course curves (D) and microphotographs (E) illustrating the sudden collapse of fluorescence observed in GadGFP-positive cells after exposure to NMDA (800 μM Int-P). Three GadGFP-positive neurons identified 1, 2, and 3 have been chosen as typical examples. (F, G) Microphotographs acquired by videomicroscopy visualizing GadGFP-positive cells and dead cells (7-AAD positive) after exposure to aCSF (F) or NMDA (G) during 50 min. Arrow heads indicate cells already dead at the beginning of the recording. Arrows indicate GadGFP-positive cells. Note that after 50-min exposure to NMDA, the green fluorescence associated with GadGFP-positive cells collapsed while a 7-AAD-positive signal appeared (G; arrows).
in the immature layers II-IV (Fig. 9A,B). Compared with NMDA, MK801 (20 μM) had the opposite effect on the mitochondrial integrity (Fig. 9B,C). At high magnification, overlay of the fluorescent signals corresponding to the aggregated (590 nm) and monomeric (530 nm) forms of JC-1 in the control condition revealed the presence of sparse cells presenting a strong fluorescent yellow signal, indicating intense mitochondrial activity (arrows, Fig. 9D). Treatment of the slices with NMDA (400 μM) increased the number of cells characterized by a strong mitochondrial integrity (Fig. 9E). In contrast, MK801 (20 μM) markedly decreased the number of cells capable of aggregating JC-1 (Fig. 9F). These descriptive data were confirmed by quantification of the 590/530 ratio (Fig. 9G).

**Effect of In Vivo Bax Repression on Apoptosis of GABAergic Interneurons in the Developing Neocortex of Mice Neonates**

Genes from the Bcl-2 family play a key role in initiating the mitochondrial apoptotic pathway (Youle and Strasser 2008). Treatment of slices with MK801 (20 μM) for 3 h increased Bax immunoreactivity in the immature layers II-IV (Fig. 9H,I). At high magnification, Bax immunolabeling was located in the cytoplasm (Fig. 9J). Quantification of Bax expression by western blot on slice extracts showed that NMDA (400 μM) slightly reduced Bax levels when compared with control, whereas MK801 (20 μM) significantly increased by 20.1% ± 4.2 the expression of the pro-apoptotic gene (P < 0.05; Fig. 9K).

Intracortical injections of *in vivo* ready siBax at P0 and P1 induced a strong reduction of Bax immunoreactivity in P2 cortical slices previously exposed to MK801 (20 μM) for 6 h (Fig. 10A,B). Hoechst counterstaining showed no difference in nucleus density between areas where Bax immunoreactivity was repressed or not (Fig. 10C-E). In contrast, a comparison of nucleus morphology at high magnification established that many nuclei in Bax-immunoreactive regions were condensed and/or fragmented (Fig. 10F,G), although, in Bax-repressed areas, the number of nuclei presenting a normal morphology was significantly increased in the immature layers II-IV (P < 0.001; Fig. 10H-J). Colabeling experiments showed that, in MK801-treated slices, most of the Bax-immunoreactive cells were also positive for FITC-VAD-FMK, a fluorescent substrate of caspases (Fig. 11A-E; arrows). Even if they were few, Bax-negative and FITC-VAD-FMK positive cells were also observed (Fig. 11A-E; arrow heads). In siBax-injected animals, although Bax immunoreactivity was substantially reduced, several Bax-negative and FITC-VAD-FMK positive cells were still observed suggesting that, for some cells, apoptosis occurred through a Bax independent process (Fig. 11F-J; arrow heads).

**Effect of Ketamine on Neuronal Death, Apoptosis, and Calcium Mobilization in the Developing Neocortex of Mice Neonates**

Ketamine, an anesthetic currently used in pediatric, exhibits NMDA antagonist properties (Orser et al. 1997). Because ketamine is often given as a chronic infusion to neonates, we investigated whether ketamine exerted harmful effects, most notably on the neurons in the immature layers II-IV. Exposure of cortical slices from mice neonates to various concentrations (10–100 μM) of ketamine alone induced a small and non-significant decrease in LDH activity (Fig. 12A). In contrast, ketamine (100 μM) combined with NMDA inhibited NMDA-induced excitotoxicity (P < 0.001; Fig. 12A). Ketamine (10-100 μM) administered alone in aCSF had no significant effect on caspase-3 activity (Fig. 12B). On the contrary, when it was coincubated with NMDA (400 μM), the anesthetic was a very potent inducer of caspase-3 activity. The pro-apoptotic effect of ketamine was significant starting at 10 μM (P < 0.05) and was marked at 100 μM (P < 0.001; Fig. 12B). Calcimetry studies performed on cortical slices at the level of the immature layers II-IV showed that MK801 (40 μM intrapipette) as well as ketamine (200 μM intrapipette) diminished the calcium mobilization induced by NMDA (800 μM intrapipette; Fig. 12C).
In addition, when compared with the control condition, co-exposure of cortical slices to ketamine (100 μM) and NMDA (400 μM) for 6 h induced a Bax-immunoreactive signal in layers II-IV (Fig. 12D-G; arrows).

Discussion

**Dual Effect of NMDA Receptor on the Immature Cortex**

Perinatal brain lesions recover different histopathological patterns depending of the gestational age at birth. In preterm infants, lesions are most frequently observed at the level of the periventricular white matter (Volpe 2009). In these lesions, late oligodendrocyte progenitors and immature oligodendrocytes (Back et al. 2007) are highly sensitive to excitotoxic stress involving both AMPA (Follett et al. 2000) and NMDA (Manning et al. 2008) glutamate receptors. In full-term infants, anoxic encephalopathy more frequently affect the gray matter causing massive neuronal death concomitantly with intense NMDA receptors activation (Inder and Volpe 2000; Mishra et al. 2001). Based on these observations, it is commonly admitted that uncontrolled release of glutamate by presynaptic neurons plays a key role in the development of neonate brain lesions through induction of excitotoxicity. Accordingly, several studies described protective effects of glutamate receptor antagonists against perinatal brain lesions in animal models (Follett et al. 2004; Manning et al. 2008; Marret et al. 2001). In agreement with these data, our results revealed that MK801 induced a small reduction of the LDH activity compared with control slices (suggesting a basal excitotoxic cell death after brain slicing) and abrogated NMDA-induced excitotoxicity in layers V-VI. Nevertheless, recent studies suggest that the NMDA-induced excitotoxicity may be due not only to massive glutamate release by the presynaptic neurons but also to the expression of specific NMDA receptor subunits by the postsynaptic neurons (Liu et al. 2007). Consistent with this hypothesis, we found that a given concentration of NMDA induced a dual effect on neonatal cortical slices: strong excitotoxicity occurred in the deep cortical layers (believed to contain postmigrating and differentiated neurons) contrasting with a strong antiapoptotic effect in the immature layers II-IV (where neurons are still migrating at this age; Berry et al. 1964). In addition, a given NMDA concentration induced sustained calcium mobilization in layer VI neurons and acute transient calcium mobilization in neurons of layers II-IV. Altogether these data suggest that kinetic properties may differ between NMDA receptors in cortical layers II-IV and those in layer VI. Consistent with this hypothesis, Liu and coworkers found that NR2A mRNAs were detected mainly in the superficial layers of the developing mouse neocortex on P2, whereas NR2B mRNAs were expressed in all layers (Liu et al. 2004). Interestingly, NR2A-containing receptors have shorter channel kinetic than do their NR2B counterparts (Barth and Malenka 2001) and NR2B-containing receptors mediate NMDA-induced necrotic death of cultured cortical neurons (Liu et al. 2007).
Bax and the Mitochondrial Pathway are Involved in the Apoptotic Effects of NMDA Antagonists in the Superficial Cortical Layers

Several groups described protective effects of NMDA receptor blockade on the neonatal rat brain (McDonald et al. 1990; Manning et al. 2008). For example, it has been shown that memantine attenuates white matter injury and death of oligodendrocytes in a model of periventricular brain damage (Manning et al. 2008). In another study, however, NMDA antagonists were shown to induce cell death in the immature cortex (Ikonomidou et al. 2000). Our data help to reconcile these apparent discrepancies, because we found a dual effect of NMDA antagonists depending on the cortical layers. Although previous studies showed that NMDA antagonists could induce apoptosis (Ikonomidou et al. 1999), the mechanisms involved were not elucidated. Several apoptotic pathways can activate the executioner caspase-3 (Siegel 2006). For example, caspase-8, which can be activated via death factor receptors, is associated with the extrinsic apoptotic pathway, whereas caspase-9 is involved in the mitochondrial-dependent apoptotic pathway. Upon activation of this last pathway, the mitochondrial integrity is disrupted and controlled by proteins of the Bcl-2 family (Youle and Strasser 2008). We found that MK801 was a potent inducer of the pro-apoptotic gene Bax, an effect that disrupted mitochondrial integrity. Because Bax has been shown to play a key role in apoptosis control during neuronal development (Zhokhov et al. 2008), we hypothesized that MK801 could induce apoptotic cell death in the superficial layers II–IV of the neonate cortex via induction of the mitochondrial apoptotic pathway. Our results showed that IETD-FMK,
a caspase-8 inhibitor, did not affect the MK801-induced stimulation of caspase-3 activity. In contrast, Z-LEHD-FMK, a caspase-9 inhibitor, blocked this stimulatory effect. Nevertheless, up to 14 caspases have been cloned to date (Siegel 2006). Although the effect of each caspase is greatest at specific peptide cleavage sites, several caspases can cleave the same peptidic sequence. Because inhibitors of caspases have been synthesized based on their cleavage sites, their specificity remains limited (Siegel 2006). Therefore, we performed in vivo siBax transfections and found that siBax markedly decreased Bax immunoreactivity, pyknotic nucleus density, and caspase activation induced by MK801. Taken together, these data indicate that MK801 promotes apoptosis in the superficial layers II–IV of the immature neocortex via Bax induction and mitochondrial pathway activation.

**GABAergic Interneurons: A Target of Glutamate Excitotoxicity and MK801-Induced Apoptosis**

In one hand, a postmortem study has recently described a loss of cortical GABAergic neurons in preterm infants presenting brain injuries, suggesting a sensitivity of this neuronal type to excitotoxicity (Robinson et al. 2007). On the other hand, previous in vitro studies indicated that glutamate is a key factor in promoting the survival of cultured immature GABAergic interneurons through a NR2A-dependent mechanism (Kinney et al. 2006; Vutskits et al. 2006, 2007). The present results
would contribute to interpret this apparent discrepancy. Indeed, in deep cortical layers, we observed that NMDA was able to induce a rapid increase of the GadGFP-positive cell volume followed by a cell loss suggesting that NMDA acts as a pro-necrotic factor for mature GABAergic cells. On the contrary, double immunostaining experiments performed on MK801-treated cortical slices revealed that several of the cleaved-caspase-3 immunoreactive cells present in the immature superficial layers II–IV had a GABAergic phenotype. There is now compelling evidence that, during cortical development, numerous GABAergic neurons are generated in the subpallial telencephalon and reach the cortex by tangential migration (Marin and Rubenstein 2001; Letinic et al. 2002). After entering the cortex, the immature GABAergic neurons reach their final destination via several routes located in the marginal zone, subplate and lower intermediate zone (Lopez-Bendito et al. 2008). Even if this process mainly occurred during prenatal life from E12 to E18, the maturation of GABAergic cells is not

Figure 10. Effect of in vivo administration of Bax siRNA on Bax immunoreactivity and nucleus condensation induced by MK801. (A, B) Visualization at low magnification of Bax immunoreactivity in cortical slices exposed to MK801 (20 μM) from 2-day-old control mice (A) and from 2-day-old mice injected with in vivo ready Bax siRNA (B). (C–E) Overlay (F) of Bax immunoreactivity (C) and Hoechst labeling (D) in Bax-immunoreactive (BIA) and Bax-repressed (BRA) areas. (F, G) Detailed visualization of the nucleus morphology (F) associated with the Bax-immunoreactive area (G). Arrow heads indicate condensed and fragmented nuclei. Arrow indicates a nucleus with normal morphology. (H, I) Detailed visualization of the nucleus morphology (H) associated with the Bax-repressed area (I). Arrow heads indicate condensed and fragmented nuclei. Arrows indicate nuclei with normal morphology. (J) Quantification of pyknotic nuclei in control cortical slices, cortical slices exposed to MK801 (20 μM) and cortical slices from in vivo ready siBax-injected mice exposed to MK801 (20 μM). One-way ANOVA test showed a group effect ($F = 26.70; P < 0.0001$) and the Tukey’s multiple comparison test indicated $*** P < 0.001$ versus control and $**** P < 0.001$ versus MK801. Each value represents the mean (±SEM) of 3 independent experiments.
totally achieved until a few days after birth (Di Cristo 2007). In light of these data, our results suggest that at P2 MK801 is pro-apoptotic for immature GABAergic neurons present in layers II–IV. Consistent with this hypothesis, Abeokawa et al. (2007) showed in a recent in vivo study that prenatal injections of MK801 reduce the cortical density of parvalbumin-positive GABAergic neurons. Otherwise, several reports demonstrated that an apoptotic cell death naturally occurs during brain development (Haydar et al. 1999). This programmed cell death contributes in the elimination of nervous cells that have not established proper connections or received adequate trophic inputs (Sherrard and Bower 2001). Consistent with these studies, invalidation of caspases such as caspase-9 resulted in a massive excess of nervous cells indicating that this apoptotic program is essential to assure a correct neurodevelopment (Kuida et al. 1998). Altogether these data suggest that exposure of immature GABAergic cells to MK801 would repress a trophic input of glutamate and induce their apoptotic death through a Bax dependent mechanism.

Ketamine Mimicked the Effect of MK801 in Layers II–IV

Although the NMDA antagonist MK801 is not administered in human neonates, ketamine is an anaesthetic with NMDA antagonist properties currently used in paediatric to reduce pain of infants (Morton 2004). Because of its bronchondilatory effect and the cardiovascular stability of patients treated with ketamine, it is sometimes administered in continuous infusion in preterm neonates (Tobias 2005). Considering all these points, we hypothesized that ketamine could mimic the action of MK801 and activate the mitochondrial apoptotic pathway in neurons located in the immature layers II–IV. In one hand, the present study showed that exposure of P2 cortical slices to ketamine, even at low concentration, was able to induce an increase of the caspase-3 activity and a stimulation of Bax expression. In addition, calcimetry experiments revealed that ketamine significantly diminished NMDA-induced calcium mobilization in neurons of the superficial layers II–IV. On the other hand, immature and migrating GABAergic neurons are still detected in the human neocortex from 23 to 25 weeks of gestation, a developmental stage which overlaps with the age of preterm infants admitted in neonatal intensive care units (Letinic et al. 2002). Taken together, these data strongly suggest that in human preterm neonates, the use of ketamine could have a deleterious effect on immature GABAergic neurons of the neocortex. In order to test this hypothesis, it would be interesting to determine if chronic exposure of pregnant mice to ketamine would have long term consequences on cortical GABAergic neurons in the offspring.

In conclusion, by using a model of cortical organotypic slices from mice neonates, the present study demonstrated that a same concentration of NMDA exerts a dual effect causing excitotoxic damages in the deep layer VI and inhibiting apoptosis in the superficial layers II–IV. These 2 effects of NMDA were associated with different characteristics of intracellular calcium mobilization. In superficial layers, where NMDA protected against apoptosis, the glutamate agonist induced acute transient calcium mobilization whereas in the deep layer VI, where NMDA was excitotoxic, there was a sustained increase in intracellular calcium levels. In addition, NMDA was pro-necrotic for GABAergic cells in the deep layer VI, whereas the NMDA antagonist MK801 was pro-apoptotic for GABAergic cells present in the immature superficial layers II–IV. This effect required activation of the mitochondrial apoptotic pathway and the expression of Bax. Ketamine, an anaesthetic currently used in infants and children reproduce
the molecular and cellular deleterious effects of MK801. Finally, because the GABAergic interneurons are not yet fully differentiated in humans before full term, caution would be in order when using ketamine in preterm babies.

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