Inhibition of Cell Cycle Pathway by Flavopiridol Promotes Survival of Cerebellar Granule Cells after an Excitotoxic Treatment

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ABSTRACT
Kainic acid (KA)-induced neuronal damage and the protective effects of flavopiridol were studied in primary cultures of rat cerebellar granule cells (CGNs). When neurons were treated with 500 μM KA, the percentage of cells with condensed nuclei measured by nuclear counting increased by up to 55%. After flavopiridol treatment, an antitumoral drug that is a broad inhibitor of cyclin-dependent kinases, the percentage of condensed nuclei decreased by up to 26%. Furthermore, this KA-mediated cell death was only partially dependent on the activation of the initiator caspase-9 and the effector caspases-3 and -6. This argues for a minor role of caspases in the intracellular pathway leading to KA-induced programmed cell death in CGNs. We examined the possible implication of cell cycle proteins in KA-induced neurotoxicity. We found an increase in the expression of proliferating cell nuclear antigen and E2F-1, two proteins implicated in S-phase, by Western blot. KA increased bromodeoxyuridine incorporation in CGNs, a marker of cell proliferation, and flavopiridol attenuated this effect. These results indicated that flavopiridol decreased the expression of cell cycle markers in CGNs after KA treatment. Flavopiridol might thus be used as a preventive agent against neurodegenerative diseases associated with cell cycle activation.

The pathogenesis of neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis is poorly understood. The exact mechanisms of neuronal death in these neurodegenerative diseases are still not clear, although it is widely accepted that excitotoxicity may be involved (Atlante et al., 2001; Mattson, 2003). Glutamate, the main excitatory neurotransmitter in the brain, mediates its effects through the activation of two classes of receptors, ionotropic and metabotropic. Ionotropic glutamate receptors are classified in three subtypes, N-methyl-D-aspartate, AMPA, and kainate receptors. Although the implication of ionotropic glutamate receptors in neurodegenerative diseases is widely accepted, the molecular mechanisms are not completely understood (Uberti et al., 2002).

Previous studies suggest that the first step in neuronal cell death is the increase in the intracellular levels of calcium (Atlante et al., 2001). Indeed the elucidation of the mechanisms by which calcium evokes the neurotoxic process is one of the challenges in neuroscience.

Various pathways have been proposed to explain the excitotoxic process. First, several studies suggest that stimulation of ionotropic glutamate receptors induces cell death through the generation of reactive oxygen species. Furthermore, antioxidants such as melatonin, vitamin E, and others may protect from excitotoxicity (Puttfarcken et al., 1993; Giusti et al., 1995; Castilho et al., 1999; Klein and Ackerman, 2003).

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone; BdrU, bromodeoxyuridine; BSA, bovine serum albumin; Ac-DEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide; Ac-LEHD-pNA, N-acetyl-Leu-Glu-His-Asp-p-nitroanilide; Cdk, cyclin-dependent kinase; CGN, cerebellar granule neuron; KA, kainic acid; LDH, lactate dehydrogenase; LH-BSA, Locke-HEPES buffer; PBS, phosphate-buffered saline; PCNA, proliferating cellular nuclear antigen; PI, propidium iodide; pRb, retinoblastoma protein; TBS-T, Tris-buffered saline/Tween 20; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride.
A second mechanism implicated in the excitotoxic process is the induction of apoptosis mediated by the activation of the intrinsic pathway. The existence of two apoptotic routes has been proposed. The extrinsic pathway is mediated by the activation of FAS receptors and the intrinsic is regulated by mitochondria (Klein et al., 2003). Mitochondria could release several proapoptotic proteins through the aperture of the mitochondrial permeability transition pore. This pathway has also been referred to as the mitochondrial cell-death pathway (Van Loo et al., 2002).

It has been reported that mitochondrial permeability transition pore also increases the permeability of the inner mitochondrial membrane, which leads to the influx of proteins with sizes less than 1.5 kDa. Cytochrome c is probably the best known apoptotic inductor that activates the cysteine proteases known as caspases, which mediate the apoptotic process. Caspase-3 is the final effector. Among the proapoptotic proteins released by mitochondria, apoptosis-inducing factor induces apoptosis by a mechanism independent of caspase activation. In fact, apoptosis-inducing factor-induced apoptosis is not prevented by z-VAD-fmk, a well established caspase inhibitor (Nicotera and Leist, 1997; Van Loo et al., 2003).

Another potential cell-death mechanism involved in excitotoxicity is the expression of cell cycle proteins (Park et al., 2000). This hypothesis is supported by the finding that in the human brain, patients with neurodegenerative diseases such as Alzheimer disease, Parkinson’s disease, amyotrophic lateral sclerosis, and epilepsy express cell cycle markers such as E2F-1, retinoblastoma protein (pRb), or cyclin D (Nagy et al., 1997; Raina et al., 2001; Jordan-Sciutto et al., 2002a,b; Ranganathan and Bowser, 2003).

Previous studies in our laboratory suggest that KA induces the expression of several proteins that are involved in the cell cycle (Verdaguer et al., 2002a,b). Inappropriate expression or activation of cell cycle proteins in postmitotic neurons has been said to evoke programmed cell death (Giardina et al., 1998, 2002; Shirvan et al., 1998; El-Khodor et al., 2003). Thus, one of the hypotheses proposed is that this pathway mediates reentry of postmitotic neurons into the cell cycle, resulting in some intracelluar signals responsible for programmed cell death. Here we attempt to elucidate the role of the induction or expression of proteins related with cell cycle progression in CGNs in KA-induced cell death. We also studied the neuroprotective properties of flavopiridol, a broad inhibitor of cyclin-dependent kinases in KA-induced neurotoxicity (Knockaert et al., 2002; Sausville, 2002).

Materials and Methods

Materials

Flavopiridol was a kind gift from Aventis (Strasbourg, France). Propidium iodide (PI), EDTA, HEPES, CHAPS, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Chromogenic substrates Ac-DEVD-p-nitroaniline, Ac-VEID-pNA and Ac-LEHD-pNA were obtained from Oncogene Science Inc. (Cambridge, UK). Cell culture media and fetal calf serum were obtained from Invitrogen (Paisley, UK). Cell culture salts and enzymes as well as Mowiol 4-88 and Triton X-100 were obtained from Sigma-Aldrich.

Chromogenic substrates Ac-DEVD-pNA and Ac-LEHD-pNA were obtained from Panreac Quimica (Barcelona, Spain).

Cell Cultures

Primary cultures of cerebellar granule cells were prepared from 7-day-old Sprague-Dawley rats by the method of Verdaguer et al. (2002b). Cerebella freed of meninges were trypsinized and treated with DNAse. Cells were adjusted to 8 \times 10^5 cells/ml and were plated on poly-L-lysine-coated 96-well plates (Falcon) at a density of 320,000 cells/cm². Cultures were grown in Eagle’s basal medium (Invitrogen) containing 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, 0.1 mg/ml gentamicin, and 25 mM KCl. Cytochrome arabinoside (10 \mu M) was added 16 to 18 h after plating to inhibit the growth of non-neuronal cells. Cultures prepared by this method were enriched in granule neurons by more than 95%.

Treatment of CGCs and Survival Assay

CGCs were used after 7 to 10 days in vitro. KA was dissolved in culture medium and adjusted to pH 7.4 with NaOH, if necessary, before being added to the cell culture. Flavopiridol was added to the medium, at the concentrations indicated, 30 min before addition of KA. Cell death was determined 24 h after KA addition using the LDH-cytotoxicity assay kit (Medical and Biological Laboratories Co., Ltd., Waterton, UK).

Analysis of Aneuploid Nuclei by Flow Cytometry

Aneuploid nuclei were measured after 24 h of KA treatment as described previously (Verdaguer et al., 2002a). In brief, the culture medium was removed; cells were collected from culture plates by pipetting and washed in phosphate-buffered saline (PBS). Flow cytometry experiments were carried out using an Epics XL flow cytometer by adding PI (10 \mu g/ml) 30 min before. The instrument was set up with the standard configuration. Excitation of the sample was carried out by using a standard 488-nm air-cooled argon-ion laser at 15-mW power. Forward scatter, side scatter, and red (620 nm) fluorescence for PI were acquired. Optical alignment was based on the optimized signal from 10-nm fluorescent beads. Time was used as a control of the stability of the measurement. Red fluorescence was projected onto a 1024 monometrical histogram. Aggregates were excluded by gating single cells by their area versus peak fluorescence signal.

Quantification of Propidium Iodide Condensed Nuclei

CGNs were grown on glass coverslips after treatment with KA (500 \mu M) alone or in the presence of flavopiridol (0.1 \mu M to 1 \mu M). After treatment, cells were fixed in 4% paraformaldehyde/PBS at pH 7.4 for 1 h at room temperature. After washing in PBS, they were incubated for 3 min with a solution of PI in PBS (10 \mu g/ml). Coverslips were mounted in Mowiol 4-88. Stained cells were visualized under UV illumination using the 20× objective (Eclipse TE 200, Nikon; Instech Corp., Kanagawa, Japan), and their digitized images were captured. Nuclei showing high fluorescence and condensed chromatin were scored by counting at least 500 cells for each sample in three separate experiments.

Measurement of Cytosolic Ca²⁺ Increase

The increase in intracellular free Ca²⁺ was determined in CGNs grown on glass coverslips (Corning Costar Corp., Acton, MA), using a Mg²⁺ -free, Locke-HEPES buffer (LH-BSA) containing 0.1% BSA, which consisted of 154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 1.3 mM CaCl₂, 5.6 mM d-glucose, 10 mM HEPES, and 0.1% BSA (pH = 7.35). After 9 to 13 days in culture, a coverslip was carefully transferred to a Petri dish containing 3 ml of LH-BSA buffer and 2 \muM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) and incubated at 37°C for 1 h in a cell incubator. For fluorescence recording, the coverslip was carefully rinsed in LH-BSA buffer, mounted on a specific holder (coverslip accessory L2250008; PerkinElmer Life and Analytical Sciences, Boston, MA), and placed in a quartz cuvette containing 1.3 ml of LH-BSA buffer. Measurements were made at
37°C with continuous mild stirring in an LS50B PerkinElmer fluorescence spectrometer equipped with a fast-filter accessory for fura-2 fluorescence ratio measurements. Emission data (510 nm) were collected with alternate excitation at 340 and 380 nm and the ratio $F_{340}/F_{380}$ calculated in real time, using proprietary software (FLWinLab 2.0; PerkinElmer).

**Evaluation of DNA Synthesis: BrdU Labeling.** The effects of kainic acid on cell proliferation were examined after 24 h of treatment. Cells were incubated with KA in the presence of flavopiridol (1 μM). We evaluated cell cycle progression by monitoring the incorporation of BrdU into the cells cultured in microtitre plates. After partial denaturation of double-stranded DNA, BrdU was detected immunochemically, which allowed us to count the cells that synthesize DNA. BrdU incorporation was measured using a colorimetric-based detection kit according to the manufacturer’s guidelines (OncoGene, Darmstadt, Germany).

**Assay of Caspase Enzymatic Activities.** We used the colorimetric substrate Ac-DEVD-p-nitroaniline for the determination of caspase-3, Ac-VEID-pNA for caspase-6, and Ac-LEHD-pNA for caspase-9. Caspase activity was measured according to the following method. At 24 h after KA treatment, CGNs were collected in a lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, pH 7.4). Then, 50 μg/μl of protein was incubated with a 200 μM concentration of the corresponding p-nitroaniline substrate in assay
buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 μM dithio-

threitol, 0.1 mM EDTA, pH 7.4) in 96-well plates at 37°C for 24 h.

Absorbance of the cleaved product was measured at 405 nm in a
microplate reader (Bio-Rad, Hercules, CA). Results are expressed as
percentages of the absorbance measured in vehicle-treated cells.

Western Blot Analysis. Aliquots of tissue homogenate, contain-
ing 30 μg of protein per sample, were placed in sample buffer [0.5 M
Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercap-
toethanol, 0.05% bromphenol blue] and denaturized by boiling at
95–100°C for 5 min. Samples were separated by electrophoresis on
12 to 15 acrylamide gels. Subsequently, proteins were transferred to
polyvinylidene fluoride sheets (Immobilon TM-P; Millipore Corpora-
tion, Bedford, MA) using a transblot apparatus (Bio-Rad). Mem-
branes were blocked overnight with 5% nonfat milk dissolved in
TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5).

Membranes were then incubated with primary mouse monoclonal
antibodies against PCNA (Oncogene, 1:100), a 37-kDa protein de-
tected in a cell cycle-dependent manner; E2F-1 (Neomarkers, 1:100),
a 46-kDa protein that acts as a transcription factor implicated in
apoptosis; and Rb, which recognizes the retinoblastoma p107 (Neo-
markers, 1:1000). After 90 min, blots were washed thoroughly in
TBS-T buffer and incubated for 1 h with a peroxidase-conjugated
anti-mouse IgG antibody (Amersham Biosciences Inc., Piscataway,
NJ). Immunoreactive protein was visualized using a chemilumines-
cence-based detection kit according to the manufacturer’s protocol
(ECL kit; Amersham Biosciences Inc.).

Immunocytochemistry against E2F-1 and PCNA. CGCs were
grown on sterile glass slides. After stimuli, cells were washed twice
in PBS and fixed in 4% paraformaldehyde/PBS, pH 7.4, for 1 h at
room temperature. Cells were preincubated for 30 min with PBS
containing 0.3% Triton X-100 and 30% normal horse serum at room
temperature. After blocking, cells were incubated with antibodies
against PCNA (Oncogene, 1:100) and E2F-1 (Neomarkers, 1:100)
onight at 4°C. Cells were then washed extensively and incubated
with secondary antibody for 1 h at room temperature. Coverslips
were thoroughly washed and mounted in Mowiol 4-88, and immu-
an AMPA/KA receptor antagonist, completely blocked the influx of $\text{[Ca}^{2+}\text{]}$.

**Flavopiridol Prevents the Nuclear Condensation Induced by KA in CGNs.** Cultured neurons were treated with 500 $\mu$M KA, and aneuploid nuclei were examined by PI staining and measured by flow cytometry. KA increased the percentage of aneuploid nuclei in CGCs (7.9 $\pm$ 0.8%, $n = 8$ control cells versus 26 $\pm$ 1.7%, $n = 9$ treated cells). The PI histogram indicates the presence of two components, one related to a diminution of PI staining (indicating a DNA fragmentation) and a peak (right) related to an increase in PI incorporation that is supporting an attempt to synthesize DNA. Flavopiridol (1 $\mu$M) significantly decreased the aneuploid component induced by KA (17 $\pm$ 1.1%, $n = 8$). Flavopiridol at this concentration also prevented the fluorescence increase of PI induced by KA (right peak).

To corroborate our flow cytometry results, we examined the nuclear morphology of nuclei by fluorescence microscopy. Nuclei of methanol-fixed cultured CGCs were visualized by PI staining. Nuclei of untreated CGCs showed typical chromatin morphology (smoothly rounded nuclei). The cultures exposed to 500 $\mu$M KA for 24 h showed a nuclear condensation.

In control cultures, the percentage of condensed nuclei was about 5.5 $\pm$ 0.2. Application of 500 $\mu$M KA increased the number of condensed nuclei (55.16 $\pm$ 3.7). The toxic effects of KA were significantly reduced by 1 $\mu$M flavopiridol (26.79 $\pm$ 1.7; Fig. 2).

**Analysis of Caspase Activation after Exposure to KA.** Additionally, experiments were carried out to determine the role of the caspase pathway in KA-induced cell death. We have demonstrated previously that CGNs deprived of serum and potassium exhibit a dramatic increase in caspase-3 activity, whereas KA induced only a 20% increase (Verdaguer et al., 2002a). In the present study, using selective chromogenic substrates for caspase-3, -6, and -9, we examined the profile of caspase activation in CGNs after 500 $\mu$M KA treatment. In agreement with our previous studies, an excitotoxic treatment in neurons induced a slight increase in cleavage of the substrates for caspase-3 (Ac-DEVD-pNA), caspase-6 (Ac-VEID-pNA), and caspase-9 (Ac-LEHD-pNA), after 24 h of KA treatment, that was reverted in the presence of flavopiridol (Fig. 3).

**Flavopiridol Prevents BrdU Incorporation Mediated by KA.** To assess the effect of KA on DNA synthesis in CGNs, we carried out several BrdU incorporation experiments. KA stimulated BrdU incorporation, indicating DNA synthesis in CGNs. This action was totally prevented in the presence of 1 $\mu$M flavopiridol (Fig. 4).

**KA Leads to Up-Regulation of E2F-1 and PCNA.** In previous studies we have demonstrated that KA induces the expression of several cell cycle proteins (Verdaguer et al., 2002a,b). Since E2F-1 is involved in cell cycle progression and programmed cell death (Hou et al., 2000; Phillips and Vousden, 2001), we studied whether their intracellular localization in CGCs after a treatment with KA modulated their expression in the presence of 1 $\mu$M flavopiridol. Western blot analysis confirmed our previous studies indicating an increase of E2F-1 expression, which was reduced when cultures were pretreated with flavopiridol (Fig. 5A). Immunocytochemical studies confirmed the increase in E2F-1 expression, and the analysis of subcellular E2F-1 distribution indicated a nuclear and cytoplasmatic localization (Fig. 5B). Concomitantly, a kainate-induced decrease in Rb protein level (the

### Results

**Flavopiridol KA-Induced Excitotoxicity in CGNs Is Not Mediated by an Interaction with the AMPA/KA Receptor.** Morphology of CGNs was assessed by phase-contrast microscopy (Fig. 1A). Untreated neurons showed round cell bodies with a clear dendritic network. Incubation with kainate (24 h, 500 $\mu$M) induced axodendritic disruption. Cell damage was reduced in the presence of flavopiridol. Pretreatment with flavopiridol significantly decreased LDH release induced by KA (Fig. 1B). Next, experiments were performed to evaluate the possible interaction of flavopiridol with the AMPA/KA receptor. Figure 1C shows that the application of KA produced a rapid and marked increase in $\text{[Ca}^{2+}\text{]}$ in CGCs preloaded with the fluorescent calcium indicator fura-2. In the presence of flavopiridol (1 $\mu$M), the intracellular calcium increase was not modified. On the other hand, GYKI 52466,
dephosphorylated form of pRb, indicating a hyperphosphorylation of this protein and the release of E2F-1) was detected by Western blot in the same samples in which E2F-1 was determined. As expected, pretreatment with flavopiridol (1 μM) recovered Rb levels (Fig. 5A).

We also evaluated the expression of the PCNA, a protein synthesized in the S phase of cell cycle and an indicator of DNA synthesis. Changes in PCNA protein after a treatment with KA expression were observed by Western blot. Again, the presence of flavopiridol returned PCNA expression to control values. When immunocytochemical studies were performed, KA induced a translocation of PCNA staining from cytoplasmic to nucleic. This migration, indicative of DNA synthesis, was reduced when cells were pretreated with flavopiridol (1 μM).

**Discussion**

The primary findings of the work are: 1) KA activates the cell cycle pathway and DNA synthesis in CGCs; 2) flavopiridol...
The cell cycle machinery in KA-induced programmed cell death. Our data indicate that excitotoxicity induced by KA in CGCs was not accompanied by a dramatic increase in the activity of caspase-3, -6, or -9 compared with other neurotoxicants. These results are in agreement with other studies that demonstrate that an excitotoxic treatment induces a programmed cell death process independent of caspase activation and is insensitive to caspase inhibitors (Verdaguer et al., 2002a,b; Bezvenyuk et al., 2003).

Several in vitro studies have demonstrated the programmed mechanism activation role of E2F1, which has been characterized as a transcription factor that induces programmed cell death after its activation, such as p53 and c-JUN (Phillips and Vousden, 2001). Additional support for the role of E2F-1 in neuronal cell death is that CGN cultures from E2F-1-deficient mice are resistant to death when challenged with potassium deprivation, a well-known neuronal death model (O’Hare et al., 2002).

It is important to emphasize that protection by flavopiridol in our experimental conditions is not complete. This finding probably indicates that other pathways are involved in the process of KA-induced neuronal cell death. In support of this, several studies suggested the overexpression of other death proteins such as p53 (Xiang et al., 1996; Uberti et al., 2000), Par-4, or C-Jun (Mattson, 2003). It seems that p53 expres-

Fig. 6. Proposed flavopiridol neuroprotective mechanisms on KA-induced cell death in CGNs.

Flow-cytometric results also suggest an attempt to enter into the cell cycle. Thus, we found an increase in PI incorporation after 500 μM KA. We confirmed these results with an experiment measuring BrdU incorporation, a well known marker of DNA synthesis. We found an increase in BrdU incorporation that was prevented by flavopiridol. These data support this hypothesis: KA, after its interaction with AMPA/KA receptors, activates an oncogenic stimulus that leads to cell death by programmed cell death.

Our studies clearly demonstrate that flavopiridol attenuates the neuronal cell death mediated by KA by a mechanism independent of the interaction with the AMPA/KA receptor. This is concluded from the fact that KA-induced entry of calcium is not blocked by flavopiridol. It has been proposed that Cdk enzymes that normally regulate cell cycle progression in neurons may participate in the modulation of programmed cell death (Giardina et al., 1998; Hidetoshi and Chiba, 2001). This hypothesis is supported by previous studies in which the death of neurons, evoked by different stimuli such as serum and potassium deprivation (O’Hare et al., 2000; Martin-Romero et al., 2001; Trinh et al., 2001), excitotoxic treatment (Giardina et al., 1998; Park et al., 2000; Verdaguer et al., 2002b), and β-amyloid (Copani et al., 1999; Caricasole et al., 2003) and DNA damage agents (Gendron et al., 2001), is regulated, in part, by activation of cyclin-dependent kinases. Furthermore, there is evidence of the expression of cell cycle-related proteins in the brain of patients who have been suffering different neurodegenerative diseases (Jordan-Sciutto, 2002a,b; Yang et al., 2001, 2003).

Our data suggest that neuroprotective effects of flavopiridol can be explained by the inhibition of cyclin-dependent kinases. Entry into the cell cycle requires activation of G1 to S phase. This process is regulated by the pRb and E2F gene families when Rb is phosphorylated by cyclin/Cdk complex release E2F-1, which induces gene expression through S phase of the cell cycle or induces programmed cell death in neurons (O’Hare et al., 2000; Smith et al., 2000; Phillips and Vousden, 2001). Among the intracellular events triggered by neurotoxic concentrations of KA, we identified an increase in E2F1 expression and a diminution in Rb levels in CGCs, and the immunocytochemistry data indicate a cytoplasmatic and nuclear localization of E2F1. Under the same experimental conditions producing cell death, pretreatment of cultures with flavopiridol inhibits Cdk activity, resulting in a diminution of KA-induced E2F-1 and PCNA expression.

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A recent study demonstrated a direct relationship between E2F1 expression and programmed cell death (Phillips and Vousden, 2001; Nahle et al., 2002). Thus, E2F1 facilitates caspase activation and coupled cell cycle activation with the programmed cell death machinery. Our data indicate that excitotoxicity induced by KA in CGCs was not accompanied by a dramatic increase in the activity of caspase-3, -6, or -9 compared with other neurotoxicants. These results are in agreement with other studies that demonstrate that an excitotoxic treatment induces a programmed cell death process independent of caspase activation and is insensitive to caspase inhibitors (Verdaguer et al., 2002a,b; Bezvenyuk et al., 2003)

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sion is critical for KA neurotoxicity, since it has been demonstrated that lack of p53 completely abolishes neuronal cell death evoked by KA (Xiang et al., 1996).

In summary, we show that KA neurotoxicity in CGNs is mediated by sequential processes involving initial activation of AMPA/KA receptors, increased intracellular calcium, and then enhanced DNA synthesis. This hypothesis is based on the increase in BrdU incorporation and the increase in PCNA expression. The programmed death mechanism mediated by KA is caspase-independent, and we propose that the transcription factor E2F-1 may be a key regulator in this excitotoxicity model (Fig. 6). The neuroprotective effects of flavopiridol could be attributed to their capacity to inhibit Cdk. Further studies are necessary to evaluate the possible connections between cell cycle protein expression and other intracellular signals.

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