Ultrastructural characterization of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-induced cell death in embryonic dopaminergic neurons

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Developing neuronal populations undergo significant attrition by natural cell death. Dopaminergic neurons in the substantia nigra pars compacta undergo apoptosis during synaptogenesis. Following this time window, destruction of the anatomic target of dopaminergic neurons results in dopaminergic cell death but the morphology is no longer apoptotic. We describe ultrastructural changes that appear unique to dying embryonic dopaminergic neurons. In primary cultures of mesencephalon, death of dopaminergic neurons is triggered by activation of glutamate receptors sensitive to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and differs ultrastructurally from both neuronal apoptosis or typical excitotoxicity. AMPA causes morphological changes selectively in dopaminergic neurons, without affecting other neurons in the same culture dishes. Two hours after the onset of treatment swelling of Golgi complexes is apparent. At 3 h, dopaminergic neurons display loss of membrane asymmetry (coinciding with commitment to die), as well as nuclear membrane invagination, irregular aggregation of chromatin, and mitochondrial swelling. Nuclear changes continue to worsen until loss of cytoplasmic structures and cell death begins to occur after 12 h. These changes are different from those described in neurons undergoing either apoptosis or excitotoxic death, but are similar to ultrastructural changes observed in spontaneous death of dopaminergic neurons in the natural mutant weaver mouse.

Keywords: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; programmed cell death; glutamate receptors; ultrastructure.

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Background

Neurogenesis of dopaminergic neurons in the mouse substantia nigra pars compacta (SNpc) is completed by the end of the second week of embryonic development.[1] Many of these neurons undergo physiologic cell death postnatally, with the morphology of apoptosis.[2–5] Yet, it appears that the number of neurons immunolabeled with tyrosine hydroxylase (a marker of dopaminergic neurons) in the SNpc, remains relatively constant between birth and adulthood,[3,6,7] suggesting that dying neurons in the SNpc are not dopaminergic. This conclusion has been challenged, because expression of tyrosine hydroxylase reaches adult levels only after the first month of life,[8] and the proportion of total neurons expressing tyrosine hydroxylase increases throughout that time,[3] indicating that phenotypic maturation extends into young adulthood. It is, thus, possible that at least a fraction of neurons undergoing apoptosis in the immediate postnatal period in the SNpc are dopaminergic but have lost expression of phenotypical markers during the cell death process.[9] Indeed, there appears to be an inverse relationship between the amount of physiological death postnatally and the adult number of dopaminergic neurons in the SNpc,[3] the latter number probably depending upon genetic,[10] as well as environmental[2,4,11,12] factors.

Physiologic death in the mice SNpc peaks at postnatal day 2[2,4] and postnatal day 14.[2,4,5] The number of neurons deleted by this process can be enhanced by early destruction of their anatomical target,[11] or by destruction of dopaminergic axons.[12] The second peak may be regulated by competitive synaptogenesis in the striatum, which peaks at postnatal day 14.[13] Natural cell death in the SNpc correlates with increased expression of caspase 3 and an increased Bax:Bcl-2 ratio, but cannot be reduced by administration

of antioxidants.[14,15] Since expression of cyclins (normally associated with onset of mitosis, but leading to apoptosis in post-mitotic cells) does not change, apoptosis is likely not triggered by an aborted mitosis.[15]

Following the time window of natural cell death, destruction of the striatum still results in cell death in the SNpc, but the morphology is no longer apoptotic,[16] suggesting a different molecular mechanism is at play. In the naturally occurring mouse mutation "*weaver*", dopaminergic neurons are generated normally,[17] but die rapidly during the postnatal period[18–21] through a non-apoptotic mechanism characterized by early cytosolic changes and severe involvement of the Golgi complex.[22] The molecular mechanism(s) underlying this form of cell death, though presumably related to the *weaver* mutation in the GIRK2 potassium channel, are not presently known.

We recently showed that embryonic dopaminergic neurons in vitro die following excess activation of the ionotropic receptor to glutamate leading to transcriptional activation of nuclear factor kappa B (NFκB) and transactivation of the oncogene p53.[23] This pathway has since been confirmed in dopaminergic neurons *in vivo*.[24] The ultrastructural changes associated with this molecular pathway have not been characterized. Developing neurons can adopt one of several different morphological types while undergoing death, which presumably reflect independent molecular mechanisms. The following types have been described at the ultrastructural level: apoptotic, excitotoxic, autophagic and non-lysosomal vesiculate.[25–27] Under specific pathological circumstances these mechanisms may combine or overlap, but the sequence of ultrastructural changes in each of these forms of neuronal death can be isolated, is stereotyped, and can be consistently identified in a given cell type when the cell death process is elicited under identical circumstances. The present paper describes the ultrastructural changes occurring in dying embryonic dopaminergic neurons during activation of ionotropic glutamate receptors, which is different from either apoptosis or typical excitotoxicity.[26,27]

Materials and methods

Culture preparation

Primary cultures of ventral mesencephalon were dissociated from fetal (embryonic day 14) rat, and maintained as previously described.[28,29] Following mechanical dissociation, cells were plated onto 35 mm Petri dishes (Mattek, Ashland, Maryland) or 24 well plates (BD Falcon, Franklin Lakes, New Jersey), coated with poly D lysine and laminin (Sigma, St Louis, Missouri). Plating medium consisted of Dulbecco Modified Eagle's Medium/HAM F12 defined medium in a 50/50 mix, supplemented with glucose (25 μM) (Sigma, St Louis, Missouri), glutamine (2 μM) (Sigma, St Louis, Missouri), and 10% horse serum (Hyclone Labs, Logan, Utah). Cultures were used after 9–11 days *in vitro* (div), when dopaminergic neurons express mature

phenotypes and were selectively sensitive to AMPA-induced injury.

AMPA-induced toxicity

These procedures have been described in detail.[23,30] Cultures were transferred to a sterile laminar flow hood, and the culture medium was replaced by thorough exchange (effective dilution < 1/1000) with 1 ml of mesencephalic culture conditioned medium alone (control), or containing 100 μM alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, Sigma, St Louis, Missouri). In selected experiments to address time of commitment to die, the conditioned medium contained also 3 μM cyclosporin A (Sigma, St Louis, Missouri) diluted from a 10 mM stock in 100% ethanol, or 100 μM z-vad fmk (Sigma, St Louis, Missouri) diluted from a 10 mM stock in DMSO. Conditioned medium is obtained from sister cultures of the same age that are not used as part of the experiment. Cells were then returned to the incubator and at the conclusion of the exposure period, fixed for immunohistochemistry (see below for fixation method). The exposure times were selected according to the purpose of the experiment, as discussed in the Results section. Briefly, in experiments aimed at identifying commitment to die sampling times were 3, 6, 12 and 20 h. In experiments aimed at describing changes before, during and after the identified time of commitment, sampling times were 2, 3 and 20 h.

Assessment of neuronal survival

Cells treated with AMPA, for 2, 3, 6, 12 or 20 h and subsequently identified by either microtubule associated protein 2 (MAP2)-immunoreactivity to identify all neurons, and/or tyrosine hydroxylase (TH)-immunoreactivity to label dopaminergic neurons, were counted directly under the microscope. Neurons were counted as positive when the cytosol and proximal dendrites were clearly filled with the chromogen. The number of microscopic fields of view (0.4 mm^2) on control slides required to count 1000 cells per well (typically 52 fields at 200X) was matched in experimental wells. Each condition was counted in four wells per experiment, and reported values represent averages of at least three experiments.

Immunoperoxidase staining

For electron microscopy, cultured mesencephalic neurons were fixed in a phosphate buffered solution containing 4% paraformaldehyde and 0.5% glutaraldehyde for 30 min at room temperature. Immunoperoxidase staining using diaminobenzidine as the chromogen (Electron Microscopy Science, Hartfield, Pennsylvania) was used to allow visualization of the immunolabel in both semithin sections prepared for light microscopy and ultrathin sections prepared for transmission electron microscopy. Cultures were immunolabeled in this fashion, dehydrated through a series of alcohols and embedded in an epon:araldite mix. After the

plastic had polymerized, individual pits were cut from the plate with a jeweller's saw and the pit wall removed with pliers. The pit bottom was then separated from the epoxy embedded culture by inserting the edge of a razor blade between the polymerized plastic and the pit bottom. Several individual samples were then separated from each pit, mounted and cut on an American Optical ultramicrotome for routine light (1.0 μm thickness) or transmission electron microscopy (90–100 nm thickness). Sections for light microscopy were counterstained with toluidine blue. At low magnifications, tyrosine hydroxylase-labeled neurons were readily identified by the presence of dark brown chromogen in the cytoplasm. Neurons were readily distinguished from glia by their size and morphology. Selected fields were imaged using a Sony DKC5000 digital camera. Ultrathin sections for electron microscopy were mounted on copper grids and stained with uranyl acetate and lead citrate. Grids were viewed and photographed on a Philips EM300 transmission electron microscope. Tyrosine hydroxylase-labeled cells were also identifiable here by the presence of the chromogen (black deposit). For three dimensional reconstructions of mitochondria in dopaminergic neurons, serial sections of intact mitochondria were obtained including an entire organelle, and volumes were calculated by drawing of the perimeter in each section, and approximated to a cylindrical volume by calculating the average diameter and adding the number of sections. All calculations were performed by a blind rater.

Immunocytochemistry

Cultures were fixed at room temperature in 4% paraformaldehyde with 0.05% glutaraldehyde in phosphate-buffered saline, permeabilized in 0.25% Triton-X 100 (Sigma, St Louis, Missouri) for 10 min, and blocked by incubation in 10% goat serum. Glutaraldehyde was excluded in those experiments not intended for electron microscopy analysis. Cultures were incubated in the primary antibody for 16 h at 4°C, followed by secondary antibodies conjugated to Alexa-488 or Alexa-568 (Molecular Probes, Eugene, Oregon), or peroxidase (Boehringer Ingelheim GmbH, Ingelheim, Germany) overnight at 4°C. Controls were stained omitting the primary or secondary antibody. Cultures were mounted in antifade preparation (Slow Fade, Molecular Probes, Eugene, Oregon) with 90% glycerol. Monoclonal or polyclonal primary antibodies were obtained from commercial sources: tyrosine hydroxylase (Pel-Freeze Biologicals, Rogers, Arizona, or Boehringer Ingelheim GmbH), microtubule associated protein 2 (Boehringer Ingelheim GmbH).

Double labeling with tyrosine hydroxylase and annexin V

Mesencephalic cultures (9–11 div) in glass bottom coverslips were stained with Alexa 568-conjugated Annexin V (Boehringer Ingelheim GmbH, Ingelheim, Germany) in a HEPES- and bicarbonate- buffered salt solution (HBBSS)

containing (in mM): 116 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.8 MgSO$_4$, 1 NaH$_2$PO$_4$, 25 NaHCO$_3$, 12 HEPES, and 5.5 D-glucose, pH 7.45 $\pm$ 0.2 for 15 min, then fixed at room temperature for 30 min in 4% paraformaldehyde, and immunostained for tyrosine hydroxylase with a mouse monoclonal antibody (Pelfreeze) and an Alexa 488-conjugated secondary (Molecular Probes, Eugene, Oregon). Cells were visualized using the appropriate wavelength with a Zeiss Axiovert 135 epifluorescence microscope (Carl Zeiss AG, Gottingen, Germany).

Quantitation of abnormal mitochondriae

Ultrathin series of sections of dopaminergic neurons were obtained in control conditions or after 2 or 3 h of treatment with AMPA. Neurons stained with tyrosine hydroxylase were selected under low magnification in random fields, and the number of grossly swollen mitochondriae (christae remnants visible inside a vacuole) was counted in one every fourth section of each cell (4–5 cells per condition) by a blind observer using a grid. The number of abnormal organelles is reported as a raw score.

Statistics and data analysis

All key comparisons were performed using sister cultures exposed on the same day with otherwise identical conditions. Experiments were repeated at least three times in separate culture platings. Comparisons were performed by one-way analysis of variance with multiple comparisons test such as the Student-Newman-Keuls' test.

Results

Dopaminergic neurons are selectively affected by excitotoxicity mediated through AMPA receptor activation.

We investigated the effect of prolonged exposure of primary cultures of ventral mesencephalon to 100 μM AMPA. Dopaminergic neurons make between 10 and 20% of the total complement of neurons in this culture system, which also contains gabaergic and glutamatergic neurons. Dopaminergic neurons can be easily and unambiguously identified by staining with tyrosine hydroxylase. After 20 h of continuous exposure to AMPA, the number of dopaminergic neurons assessed by staining with tyrosine hydroxylase immunoreactivity is reduced to half, whereas other neuronal phenotypes, as assessed either by phase contrast (not shown) or by staining with microtubule associated protein 2 (MAP2) immunoreactivity are not affected (Figure 1a). A decrease in the number of tyrosine hydroxylase-stained neurons is not necessarily due to cell death, because a marked reduction in protein expression could potentially result in the same alteration in counting. To evaluate this possibility we labeled cultures with propidium iodide (PI) and bisbenza-

Figure 1. Specificity, time course and time of commitment to die during AMPA-induced neuronal death. Panel (a) shows counts of primary mesencephalic cultures (div 9-11) double stained with MAP2 and TH after 20 h of treatment with either conditioned medium (0) or 100 μM AMPA (100). Error bars represent SEM of at least 3 experiments, each including four wells per condition. ** : $p < 0.001$ compared to 0. Panel (b) shows the time course of neuronal death in mesencephalic cultures plated in gridded glass coverslips and exposed to 100 μM AMPA for the specified length of time, and stained with bisbenzamide (BB) and propidium iodide (PI). Error bars represent SEM of 3 different experiments. Panel (c) shows the results of identical cultures those of panel (b), but labeled with Annexin V, and immunostained for TH. The percentage of TH positive neurons that were also labeled with Annexin V is displayed. Error bars represent SEM of 4 different experiments. Panel (d) shows the results of an experiment stained as in panel (c), but restricting the AMPA exposure to 100 μM for 3 h. In this case individual dishes were exposed to conditioned medium (control), AMPA alone, or in combination with 3 μM cyclosporin A (CSA). Error bars represent the SEM of four experiments (each experiment consisted of three wells per condition). ** $p < 0.01$ compared to control
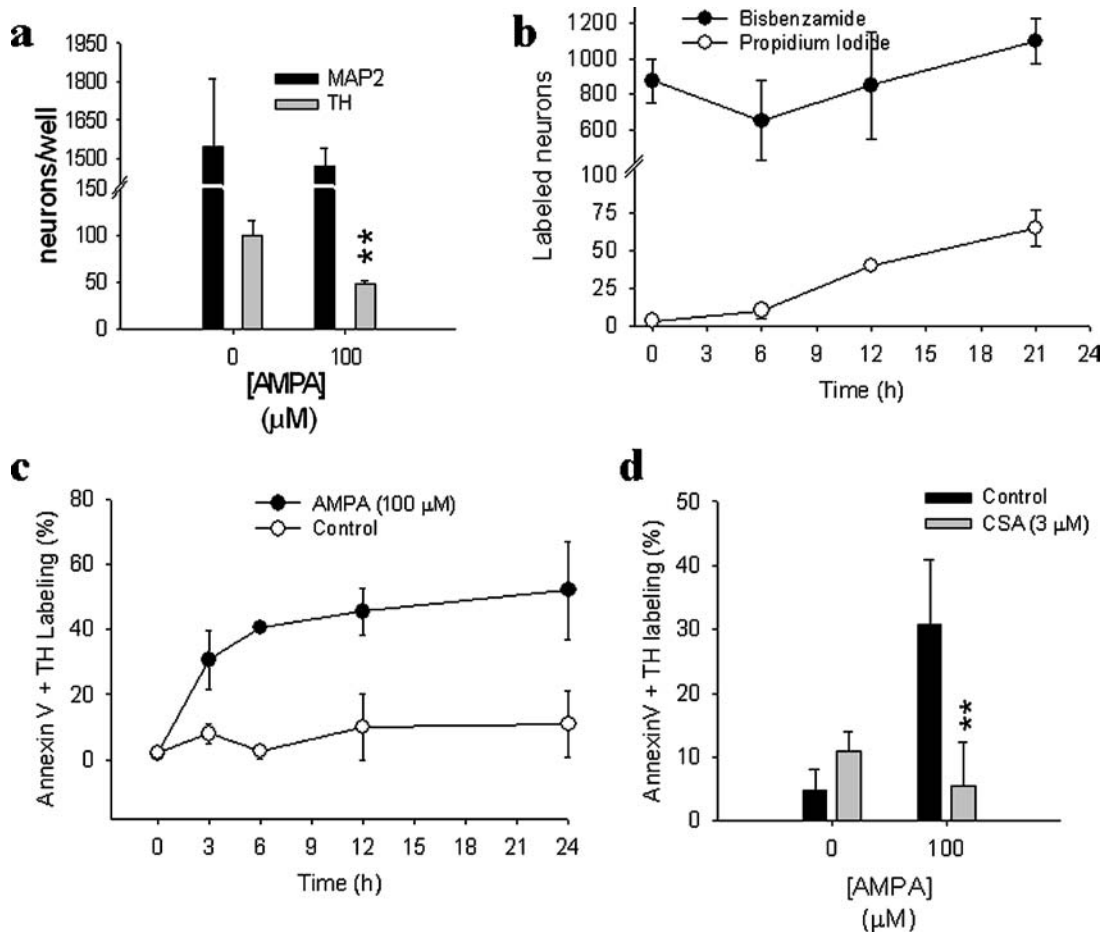
mide (BB) at 3, 6, 12, and 20 h following continuous exposure to AMPA. PI does not permeate membranes, and only labels nuclei of cells that are either death or severely damaged, whereas BB, which does permeate cell membranes, labels all nuclei. BB allows identification of glia and neurons based on the staining characteristic of each cell type. We observed an increase in PI uptake at 20 h in proportion to the loss of tyrosine hydroxylase stained neurons (Figure 1b). No significant PI uptake was observed before 12 h. Furthermore, surviving tyrosine hydroxylase stained neurons showed marked reductions in the size and complexity of neurites (Figure 2a–c), and the uptake of ^{3}H dopamine by the cultures is significantly reduced, suggesting that they are also undergoing the effects of toxicity (unpublished observation). The ultrastructural appearance of neighboring dopaminergic and non dopaminergic neurons sectioned at the level of the nucleolus is shown in figure 2d–f. In control conditions dopaminergic and non-dopaminergic neurons have similarly normal cytosol, and regular distribution of nuclear chromatin (Figure 2d). Dopaminergic neurons are easily identified by the dark deposits of tyrosine hydroxylase-bound chromogen in the cytoplasm. After 3 h of continuous exposure to 100 μM AMPA the cytosol in dopaminergic neurons is grossly altered, and the nucleus has invagination of the nuclear envelope with occasional irregular clumps of chromatin, whereas the non-dopaminergic neuron remains normal (Figure 2e). By 20 h of exposure to AMPA (Figure 2f), the cytosol is no longer evident in most dopaminergic neurons, and in the remaining ones no organelles can be identified. Nuclear changes also become more prominent.

Figure 2. AMPA toxicity in rat ventral mesencephalic cell cultures is largely restricted to DA neurons. Light microscopy and electron microscopy of cell cultures treated with 100 μM AMPA for 0 (a and d), 3 (b and e), and 20 (c and d) hours and stained with TH and diaminobenzidine demonstrates the selective nature of AMPA toxicity to dopaminergic neurons. Panels a-c show progressive (and selective) loss of TH stained neurites. Electron micrographs reveal changes including nuclear invagination, chromatin condensation, and extensive cytoplasmic vacuolation in immunostained neurons. TH-stained neurons appear normal at 0 h (d) nuclear and cytoplasmic changes appear by 3 h (e) and progress significantly with 20 h (f) of AMPA exposure. The nuclei of the non dopaminergic neurons appear normal. Scale bars = 10 μm
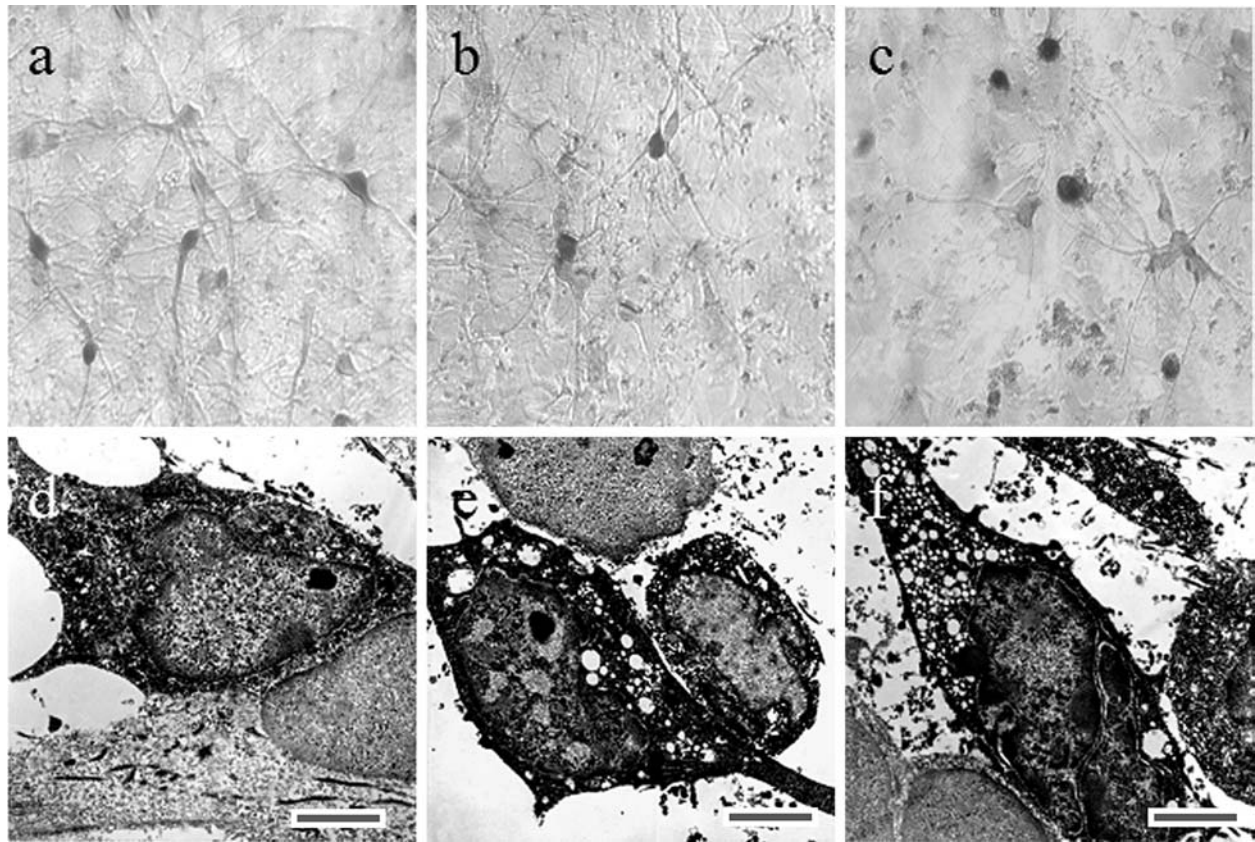
These changes are discussed in greater detail in the following paragraphs.

## Commitment to die occurs after 3 h of exposure to AMPA.

We evaluated time of commitment to die in dopaminergic neurons in several different ways. First, we measured annexin V binding by dopaminergic neurons (identified post-hoc by labeling with tyrosine hydroxylase immunoreactivity) at 3, 6, 12 and 20 h exposure to 100 μM AMPA (Figure 1c). Phosphatidyl serine translocation preceded the uptake of PI (see above, Figure 1b) by several hours, and became significantly different from controls by 3 h. Second, we studied the effect of 3 μM cyclosporin A (a treatment that effectively prevents AMPA-induced cell death), and 100 μM z-vad fmk (a caspase inhibitor that prevents apoptosis but is not effective in preventing AMPA induced injury to dopaminergic neurons), on the rate of translocation of phosphatidyl serine in dopaminergic neurons after 3 h of exposure to 100 μM AMPA. We reasoned that if phosphatidyl serine signaled

commitment to die in this cell pathway, as it does in apoptosis, cyclosporin A would prevent the loss of membrane asymmetry where z-vad fmk would not. As predicted, cyclosporin A resulted in complete prevention of translocation of annexin V (Figure 1d), but z-vad fmk did not (not shown).

AMPA causes early nuclear invagination and irregular aggregation of chromatin selectively in dopaminergic neurons.

Given the occurrence of commitment to die by 3 h, we analyzed ultrastructural changes in dopaminergic neurons labeled with tyrosine hydroxylase immunoreactivity at 2, 3 and 20 h of continuous exposure to 100 μM AMPA, and compared them to non dopaminergic neurons in the same culture dishes. The changes in the sampling protocol reflect our interest in monitoring changes before (2 h) as well as after (20 h) the putative commitment point (3 h). The results of this analysis are shown in Figures 3–5. Nuclear changes are characterized by progressive nuclear envelope invagination and irregular aggregation of chromatin

Figure 3. Time course of ultrastructural changes in the nucleus of dopaminergic neurons during AMPA-induced toxicity. Deep nuclear invagination is minimal or absent in control neurons (a) of TH-stained mesencephalic cell cultures examined by electron microscopy at 3000×. Nuclear membrane invaginations become much more pronounced at 3 h (b) and continue to increase at 20 h (c) of AMPA treatment. Nuclear perimeter measurements show a significant increase at 3 and 20 h as compared to control neurons (d).
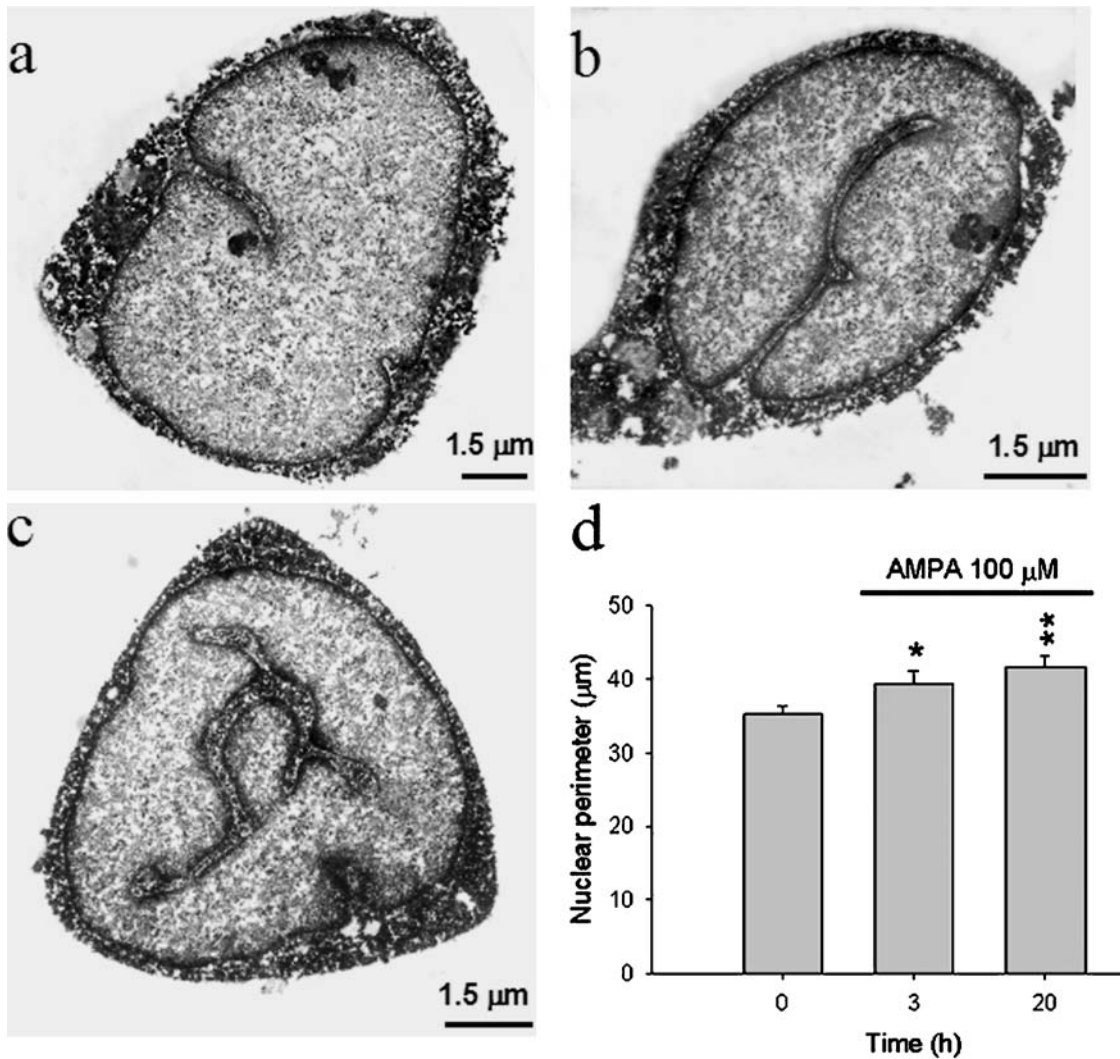
(Figure 3), which correspond to pycnotic appearance on light microscopy with Nissl staining (not shown). Quantification of the total length of nuclear envelope in sections cut through the nucleolus revealed that invagination correlated with a significant increase in its total length (Figure 3). Notably, the nuclear envelope remained intact until very advanced disintegration was apparent in the cytosol.

Commitment to die is temporally associated with changes in mitochondria and the Golgi apparatus.

Mitochondrial swelling was apparent in dopaminergic neurons by 2 h, and distorted mitochondria with disrupted christae was a frequent finding by 3 h. Stereological quantification of the frequency of these abnormal organelles revealed that they were significantly more frequent at 3 h than in control conditions, or than in non dopaminergic neurons in the same culture dish (Figure 4d). Moreover,

volumetric serial reconstructions of normal appearing mitochondria revealed that at 3 h even these were significantly enlarged when compared to control conditions in dopaminergic neurons (Figure 4e). In addition, swelling of the cisternae of the Golgi apparatus was apparent by 2 h, and very prominent by 3 h of continuous exposure to 100 μM AMPA (Figure 5). After 3 h of treatment, the finding of enlarged cytosolic, membrane bound vesicles in the vicinity of the Golgi apparatus was commonplace in dopaminergic neurons, but seldom seen in unlabeled neurons. At 20 h, the cytosol of dopaminergic neurons was shrunk and organelles were no longer recognizable (Figure 3).

Discussion

In this paper we extend our previous observations on the mechanism of cell death in embryonic dopaminergic

Figure 4. Time course of ultrastructural changes in the mitochondria of dopaminergic neurons during AMPA-induced toxicity. Mitochondria appear normal under control conditions (a). Mitochondrial swelling is evident at 2 (b) and 3 (c) hours following AMPA exposure. We counted the number of swollen, distorted mitochondria in ultrathin sections of dopaminergic neurons in control conditions or after 2 or 3 h of exposure to 100 μM AMPA. Bars represent the average number of damaged mitochondria in at least 5 cells. $*p < 0.05$. Volume estimates by serial reconstruction and area measurements of mitochondria reveals a volume increase in dopaminergic neurons exposed to AMPA (e). Scale bars = 500 nm.
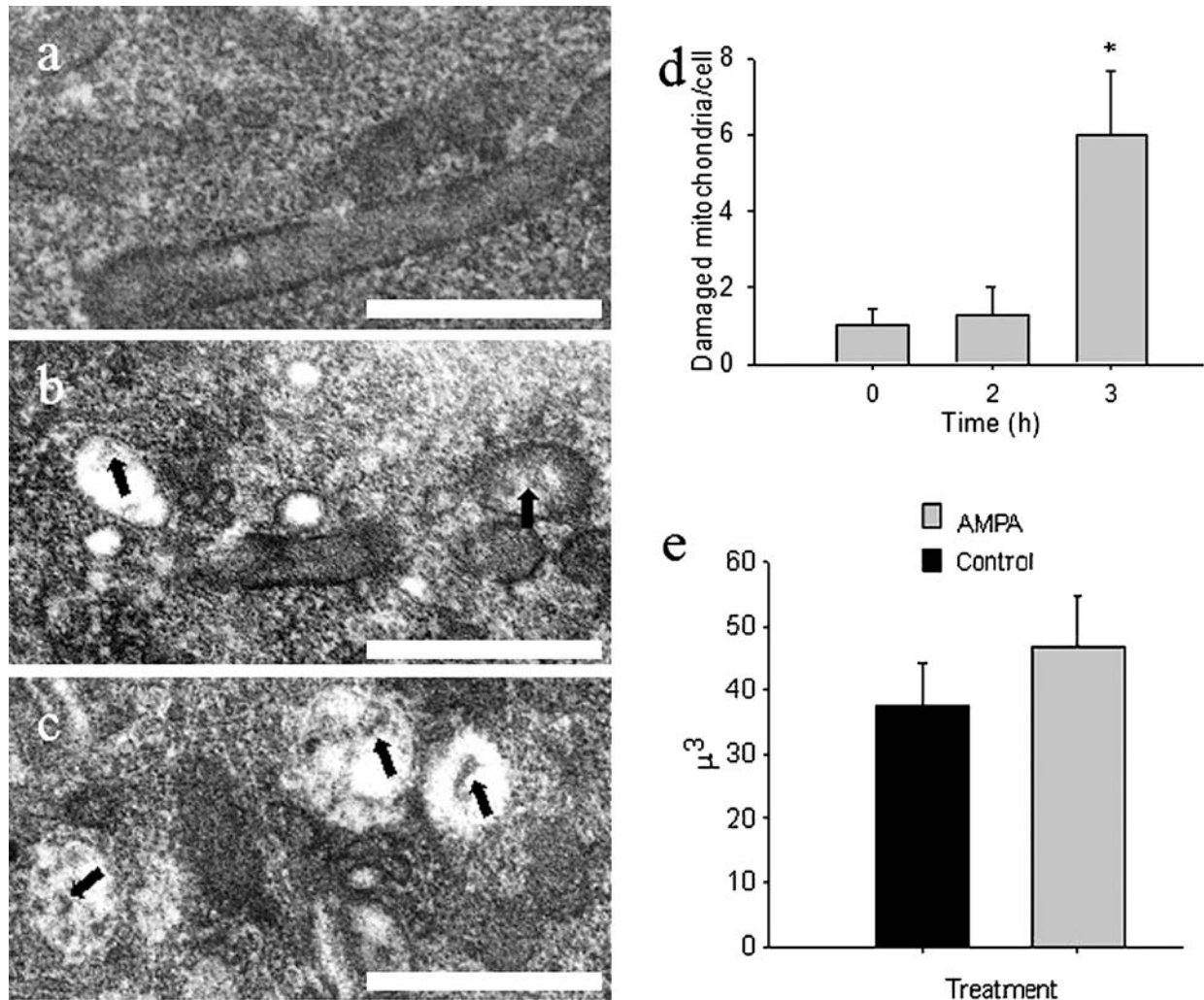

neurons. The primary aim of the present work was to characterize the ultrastructural changes associated with selective death of dopaminergic neurons induced by AMPA. Major findings include first, that following AMPA receptor activation the loss of membrane asymmetry in dopaminergic neurons, as assessed by phosphatydil serine labeling, can be prevented by cyclosporin A but not by caspase inhibition, and second, that preceding loss of membrane asymmetry, dopaminergic neurons undergo swelling of Golgi apparatus, mitochondrial distortion, and nuclear membrane invagination. Notably, each and every change observed in this cultured cells is restricted to dopaminergic neurons.

The first finding suggests that in spite of the involvement of a mitochondrial trigger (opening of a mitochondrial permeability transition pore) commitment to die of dopaminergic neurons following AMPA receptor activation

is not caspase dependent. Commitment to die is detectable by either loss of membrane asymmetry (Figure 1), or by swelling of mitochondria and Golgi complex vesicles and cistern (Figures 4–5). Since we confirmed, using a different set of methods, a previous observation that onset of death does not occur until several hours following commitment to die,[23] the cytosolic changes occurring earlier must be part of the process leading to (as opposed to the result of) neuronal death.

Our previous work has shown that AMPA receptor activation leads to depolarization, calcium influx through voltage gated channels leading to calcium-induced calcium release and loss of calcium homeostasis[29]; opening of a mitochondrial permeability transition sensitive to cyclosporin A leads in its stead to transcriptional activation of NFκB, transactivation of the oncogene p53 and neuronal

Figure 5. Time course of ultrastructural changes in the Golgi complex of dopaminergic neurons during AMPA-induced toxicity. Morphological changes in the ultrastructure of the Golgi complexes in control conditions (a), or after treatment of mesencephalic cultures with 100 μM AMPA for 2 h (b), or 3 h (c) showing dilated cisternae. Scale bars = 200 nm.
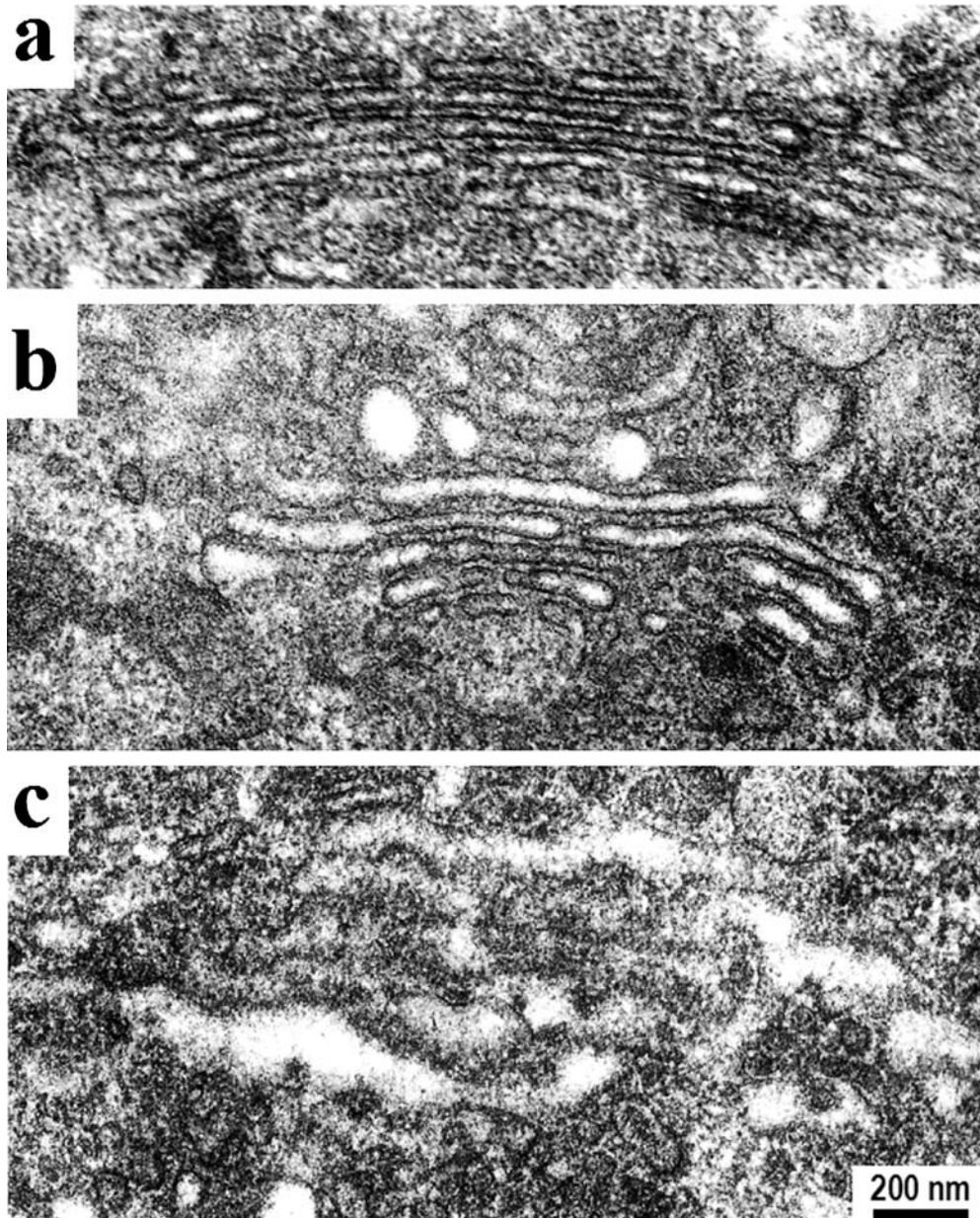
death.[23] Thus, the early involvement of mitochondria— manifested as marked swelling— is consistent with the molecular pathway. Mitochondrial changes peak by three hours, whereas nuclear changes continue to progress up to 20 h later. On the other hand, it is more difficult to interpret what the involvement of the Golgi complex, a most prominent ultrastructural change, may represent. In striatal neurons undergoing hypoxic-ischemic damage the Golgi complex is an early target, and its involvement is characterized by swelling and extensive tyrosine nitration occurring before mitochondrial failure.[31] Since glutamate excitotoxicity is known to contribute to hypoxic-ischemic damage to neu-

rons, it seems plausible that involvement of the Golgi apparatus may represent an early target of more than one form of excitotoxicity. However, the changes described in striatal neurons and those seen in cortical neurons suffering excitotoxicity differ from those described here in that cytosolic vacuolation in glutamate excitotoxicity appears more diffuse, and mitochondrial involvement appears to be a later phenomenon, occurring as late as 6 h after onset of toxicity in striatal neurons[31] and only inconsistently in cortical neurons.[26] Remarkably, the nuclear envelope in dopaminergic neurons remains intact even when cytosolic structures are no longer recognizable, a feature that distinguishes this

process from other forms of excitotoxicity.[26] The changes described in this report are consistent with those named by others as paraptosis[32] in that the onset of the death process occurs with vacuolization in the cytosol and is associated with irregular chromatin aggregation, but the receptor pathway described here, time of commitment to die, typical nuclear changes, and specificity to the dopaminergic phenotype are all novel features that differ from those described previously.[32]

It has been shown that the mechanism of natural cell death in developing dopaminergic neurons is apoptotic, but it becomes non apoptotic after synaptogenesis is complete.[4,16,33] Moreover, neuronal death occurring late in development in genetically susceptible *weaver* mice is also non-apoptotic, and has ultrastructural characteristics very similar or identical to those described here.[22] The molecular mechanism of cell death in *weaver* mice has not been completely characterized, but the phenotype is known to be caused by a single amino acid mutation in a G-protein coupled, inwardly rectifying K+ channel, GIRK2.[34] The mutation affects the channel-forming segment of the protein, causing loss of ion selectivity[35] and loss of the inward-rectifying properties of the channel.[36] The net effect of these changes in channel function is neuronal depolarization, leading to accumulation of intracellular calcium[37] and death of susceptible neurons. Both of these signaling steps, partial depolarization and accumulation of intracellular calcium with loss of calcium homeostasis, are common to neuronal death induced by AMPA in dopaminergic neurons.[23,29] In addition, the ultrastructural changes in dopaminergic neurons undergoing cell death triggered by either the *weaver* mutation or AMPA induced neuronal death are also very similar to each other and different from those seen in typical neuronal apoptosis. Indeed, in both cases cytosolic changes occur early, and the integrity of the nuclear envelope remains intact late in the process (Figures 3–5, 38). Moreover, chromatin aggregation is irregular, unlike in typical neuronal apoptosis.[26,27] Therefore, it seems probable that the final common pathway for dopaminergic neuronal death triggered either by the *weaver* mutation or by AMPA induced death may be shared.

The specificity to dopaminergic neurons of the death mechanism described here may be important to understand the susceptibility of this neuronal phenotype in Parkinson's disease.

Acknowledgments

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