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Calbindin-D28K prevents drug-induced dopaminergic neuronal death by inhibiting caspase and calpain activity

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ABSTRACT

Calbindin-D28K protects against apoptotic and necrotic cell death; these effects have been attributed to its ability to buffer calcium. In this study, we investigated the mechanisms underlying the neuroprotective effects of calbindin-D28K in staurosporine (STS)-induced apoptosis and 1-methyl-4-phenylpyridinium (MPP^+)-induced necrosis. Treatment of the dopaminergic neuronal cell line MN9D with STS or MPP^+ induced cell death that was associated with increased levels of free intracellular calcium. However, only MPP^+-induced death was inhibited by co-treatment of the cells with a calcium chelator or a sodium/calcium antiporter inhibitor. Overexpression of calbindin-D28K prevented MPP^+-induced MN9D cell death, which occurs in the absence of any detectable caspase activation. These pro-survival effects of calbindin-D28K were associated with the inhibition of calcium-mediated calpain activation, as determined by processing of Bax. Overexpression of calbindin-D28K also blocked STS-induced MN9D death. However, this effect was accompanied by the inhibition of capase-3 cleavage, poly(ADP-ribose)polymerase cleavage, and caspase activity. These findings suggest that calbindin-D28K protects against both types of cell death by inhibiting caspase- or calcium-mediated death signaling pathway.

Calcium has been implicated in the control of various neuronal processes, including excitability, transmission, differentiation, survival, and death [1]. Many classes of neurons express calcium-binding proteins that have a common structural domain known as the EF-hand [2]. Calcium-binding proteins maintain calcium homeostasis by buffering excessive intracellular levels of free calcium $[Ca^{2+}]_i$. Calbindin-D28K belongs to the EF-hand family of these proteins [3] and is abundant throughout the central nervous system [4]. Several reports have indicated that calbindin-D28K has neuroprotective effects in ischemic and glutamate toxicity models, primarily due to its ability to chelate calcium [5–7]. Recently, biochemical evidence has suggested that in osteoblasts, calbindin-D28K binds directly to caspase-3 and inhibits its activity. Therefore, it is possible that calbindin-D28K is able to prevent cell death through more than one mechanism [8].

Previously, we demonstrated that staurosporine (STS) induces caspase-dependent neuronal cell death that is morphologically and biochemically typical of apoptosis [9]. In contrast, the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) induces caspase-independent and calpain-dependent necrotic cell death in the MN9D dopaminergic neuronal cell line, and also in primary cultures of mesencephalic and cortical neurons [10–13]. In order to test the hypothesis that calbindin-D28K intervenes in more than one death pathway to promote neuronal survival, we investigated the protective effects of calbindin-D28K in MN9D dopaminergic neuronal cells exposed to calcium-dependent or calcium-independent death stimuli. In this study, we provide evidence that calbindin-D28K protects neuronal cells against STS-induced (calcium-independent) and MPP^+-induced (calcium-dependent) death via two separate mechanisms.

Materials and methods

Cell culture. MN9D cells stably overexpressing calbindin-D28K (MN9D/Calbindin) and the expression vector alone (MN9D/Neo) have been established [14]. Neither the MN9D parental cells nor the MN9D/Neo cells expressed detectable levels of endogenous calbindin-D28K as determined by immunoblot analysis [14]. Cells were seeded at a density of 2×10^4 cells in 48-well plates (Costar) or 1×10^6 cells in P-100 dishes (Corning) that had been coated with 25 μg/ml poly-D-lysine. Cultures were then maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 500 μg/ml G418 (Life Technologies; complete culture medium, CCM) for 3 days in an atmosphere of 10% CO_2 at 37 °C. Cells were subsequently switched to serum-free N2 medium [15] or serum-free N2 medium containing 1 μM staurosporine (STS; Sigma) or 50 μM 1-methyl-4-phenylpyridinium (MPP^+; RBI). In a subset of experiments, the serum-free medium also contained 40 μM BAPTA, 4 μM CGP 37157, or 50 μM calpeptin (all from Calbiochem).

MTT reduction assay. Following drug treatment, cells grown in 48-well plates were subjected to a MTT reduction assay to assess cell survival. MTT reduction assays were performed as previously described [10]. Cell survival after each treatment was expressed as a percent of survival in the untreated control (100% survival).

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Cytosolic calcium measurement. Intracellular calcium levels were measured using Fura-2 fluorescence videomicroscopy. Cells grown in 35 mm glass-bottom dishes coated with poly-D-lysine were incubated with 5 μM of the cytosolic calcium indicator, Fura-2/AM at room temperature for 30 min and with HEPES-buffered salt solution for an additional 30 min. Cells were imaged at room temperature using a Leica TCS NT system confocal microscope (Leica, Heidelberg, Germany). Images of the Fura-2 (ex: 340–380 nm; em: 510 nm) ratio were acquired with a LSM 510 camera (Carl Zeiss).

Immunoblot analysis. Following drug treatment, cells were washed with ice-cold PBS and lysed for 10 min in a buffer containing 50 mM Tris (pH 7.0), 2 mM EDTA, 1% Triton X-100, 2 mM PMSF, and 10 μg/ml leupeptin and aprotinin. Cellular lysates were centrifuged at 13,000*g* for 15 min at 4 °C. Proteins in the supernatant were measured using a Bio-Rad protein assay reagent. Forty micrograms of protein from each sample were separated by a 10–12.5% SDS–PAGE. The proteins were then electroblotted onto PVDF membrane (Bio-Rad). The membranes were then probed with the following primary antibodies: rabbit anti-calbindin-D28K (1:5000; Swant, Bellinzona, Switzerland), rabbit anti-cleavage products of caspase-3 (1:1000; Cell Signaling), mouse monoclonal anti-poly-(ADP-ribose)-polymerase (1:5000; Enzyme System Products), and rabbit anti-Bax antibody (1:3000; a generous gift from Dr. J.C. Reed). Following washing and incubation of the membranes with the appropriate HRP-conjugated secondary antibodies (1:3,000; Amersham Bioscience), specific bands were detected using enhanced chemiluminescence (ECL; Amersham Bioscience).

Fluorogenic caspase substrate assay. Cellular lysates were prepared in 50 mM Tris (pH 7.0), 2 mM EDTA, and 1.0% Triton X-100. Lysates (10 μg) were incubated with 25 μM Ac-DEVD-AMC (Calbiochem) for 1 h at 37 °C in buffer containing 100 mM HEPES (pH 7.4), 10% sucrose, 5 mM dithiothreitol, and 0.1% CHAPS. The production of the fluorescent cleavage product was monitored at Ex380/Em460 nm using a FL 600 plate reader (Bio-Tek). In separate experiments, the direct interaction between calbindin-D28K and caspase-3 was investigated by incubating 5 μg of purified caspase-3 ±2 μg of purified mouse calbindin-D28K in buffer containing 0.5% Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl, and 20 mM dithiothreitol. Reactions were incubated at 37 °C for 1 h before assaying the cleavage products.

Statistics. Data were analyzed using one-way ANOVA and a *post hoc* Student's *t* test. All values are reported as means ± SEM for the indicated number of experiments. Values of $p < 0.05$ were considered significant.

Results

We previously demonstrated that MN9D dopaminergic neuronal cells undergo caspase-dependent apoptosis within 24 h of exposure to STS, whereas treatment with the dopaminergic neurotoxin, MPP^+ induces caspase-independent and calpain-dependent necrotic cell death within 48 h [9–13]. In the present study, we

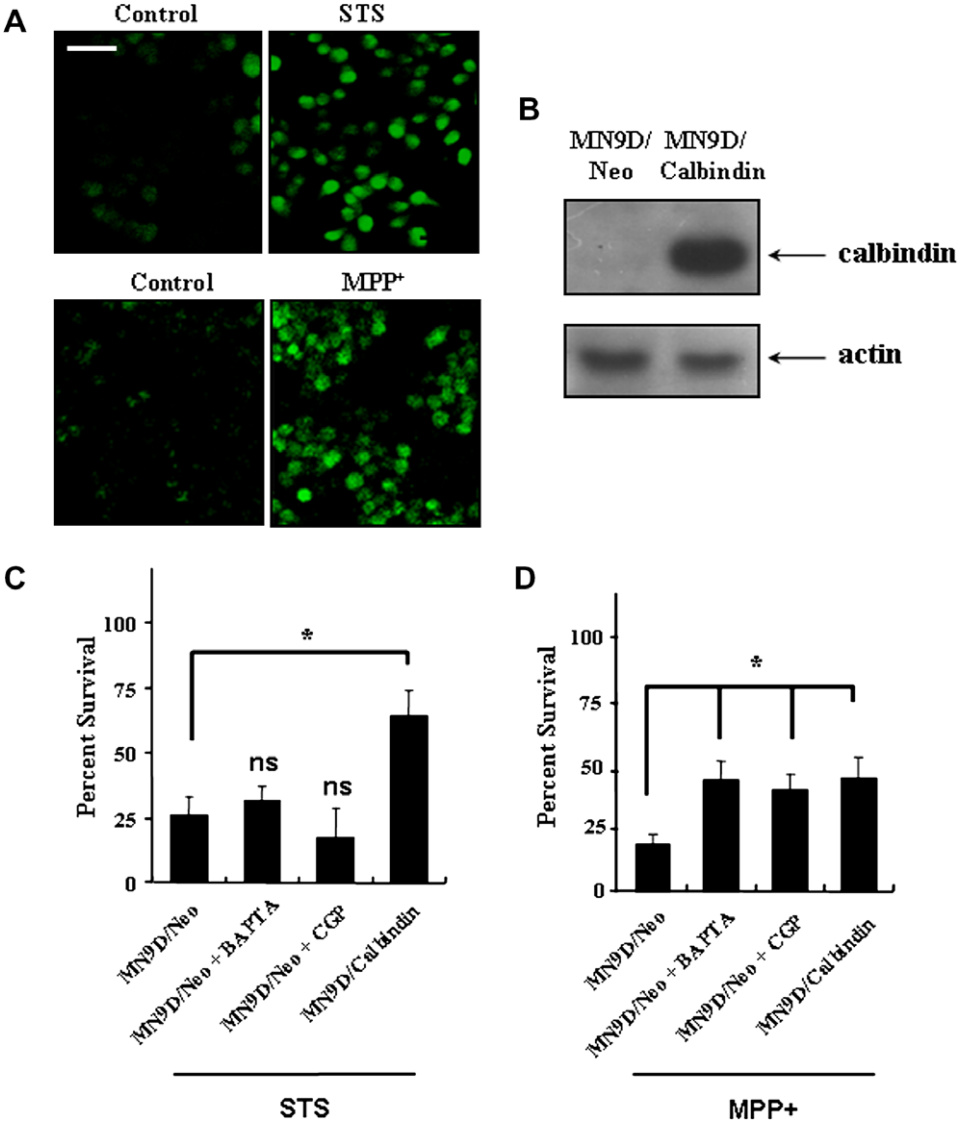

Fig. 1. Overexpression of calbindin prevents drug-induced cell death. (A) Fura-2 staining of MN9D cells treated with 1 μM STS for 1 h or with 50 μM MPP^+ for 24 h. Scale bar, 50 μm. (B) Immunoblot analysis of calbindin-D28K in MN9D cells stably transfected with chick calbindin-D28K (MN9D/Calbindin) or vector (MN9D/Neo). (C, D) MTT reduction assay of MN9D/Neo and MN9D/Calbindin cells following (C) 24 h of STS exposure or (D) 48 h of MPP^+ exposure, in the presence or absence of 40 μM BAPTA or 4 μM CGP37157. Values were expressed as a percentage relative to the untreated control (100%). All experiments were repeated 3–5 times in triplicate. $^*p < 0.05$; ns, not significant.

investigated whether the cell death induced by STS and MPP^+ is calcium dependent and whether overexpression of calbindin-D28K can prevent drug-induced cell death. As shown in Fig. 1A, treatment with both 1 μM STS and 50 μM MPP^+ increased $[Ca^{2+}]_i$, as determined by Fura-2 fluorescence videomicroscopy. The drug-induced $[Ca^{2+}]_i$ surge was largely blocked by treatments with BAPTA (a cytosolic calcium chelator), treatment with CGP 37157 (a sodium/calcium antiporter inhibitor), or overexpression of calbindin-D28K (data not shown). In order to determine whether the drug-induced $[Ca^{2+}]_i$ surge contributed to cell death, we tested the effects of BAPTA or CGP 37157 on MPP^+- and STS-induced MN9D cell death. Treatment with BAPTA or CGP 37157 significantly blocked MPP^+-induced death (Fig. 1D), but not STS-induced cell death (Fig. 1C), as determined by a MTT reduction assay. The cell death induced by both of these agents was significantly blocked by overexpression of calbindin-D28K (Fig. 1B–D). These results suggest that the mechanisms underlying the neuroprotective effects of calbindin-D28K differ for calcium-dependent (MPP^+-induced) and calcium-independent (STS-induced) cell death.

Bellido et al. [8] reported that calbindin-D28K binds directly to caspase-3 and blocks its activity in osteoblasts. We used fluorogenic caspase assays to show that exposure to STS caused a time-dependent increase in caspase activity in MN9D/Neo cells. In contrast, no increase in caspase activity was detected in cells overexpressing calbindin-D28K (Fig. 2A). Consistent with these findings, poly(ADP-ribose)polymerase (PARP) and caspase-3 cleavage products were not detected in STS-treated MN9D/Calbindin cells (Fig. 2B and C). STS-induced caspase activation in MN9D/Neo cells was not blocked by BAPTA (Fig. 2A), thus excluding the possibility that STS-induced increases in $[Ca^{2+}]_i$ mediate caspase activation. In order to further confirm that calbindin-D28K inhibits STS-induced caspase activation, we performed caspase-3 activity assays with lysates from STS-treated MN9D/Neo cells in the presence or absence of purified calbindin-D28K protein. As shown in Fig. 3A, addition of calbindin-D28K significantly blocked STS-induced caspase activity. A similar result was obtained when purified caspase-3 was incubated with purified calbindin-D28K (Fig. 3B). Taken together, these data suggest that the protective action of calbindin-D28K in STS-induced cell death is independent of its calcium-chelating activity and is probably related to direct inhibition of caspase activity.

We previously reported that treatment of MN9D cells or primary cultures of mesencephalic neurons with various concentrations of MPP^+ induces calcium-mediated calpain activation, but not caspase activation [10–13]. Co-treatment of MN9D cells with a calpain inhibitor significantly blocks MPP^+-induced cell death and the calpain-mediated cleavage of Bax [12] and fodrin (Supplemental Figure 1). Wood and Necomb [16] previously demonstrated that cleavage of Bax enhances cell death. In order to confirm whether calbindin-D28K inhibits calcium-mediated calpain activation after exposure to MPP^+, we analyzed Bax cleavage in MN9D/Neo cells that had been co-treated with BAPTA or CGP 37157. As shown in Fig. 4A, MPP^+-induced cleavage of Bax was blocked in the presence of BAPTA and CGP 37157. MPP^+-induced Bax cleavage was also inhibited by the calpain inhibitor calpeptin (Fig. 4B, lanes 4–5) or by overexpression of calbindin-D28K (lanes 6–7). The ability of calbindin-D28K to inhibit calpain-mediated cleavage of Bax indicates that the protective role of calbindin-D28K in MPP^+-induced cell death is primarily due to its ability to chelate calcium.

Discussion

Calcium has been implicated as a death-inducing factor in several neurological disorders, including Parkinson's disease (PD). Mitochondria from PD patients have a decreased ability to seques-

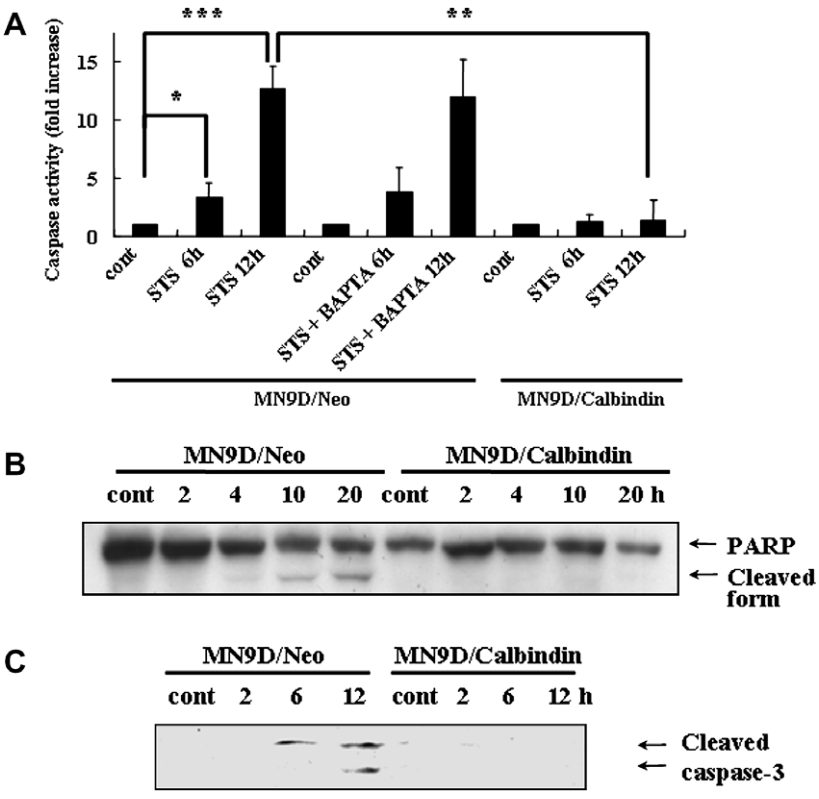

Fig. 2. Calbindin-D28K prevents the caspase activation associated with STS-induced cell death. (A) STS-induced fluorogenic caspase-3 activity in MN9D cells overexpressing calbindin-D28K (MN9D/Calbindin) or co-treated with BAPTA. Control cells (cont) were maintained in N2 medium. (B and C) Effect of calbindin-D28K overexpression on STS-induced (B) PARP cleavage and (C) caspase-3 cleavage as determined by immunoblot analysis. Values in (A) are expressed as a fold increase relative to the untreated control. All experiments were repeated three times in triplicate. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

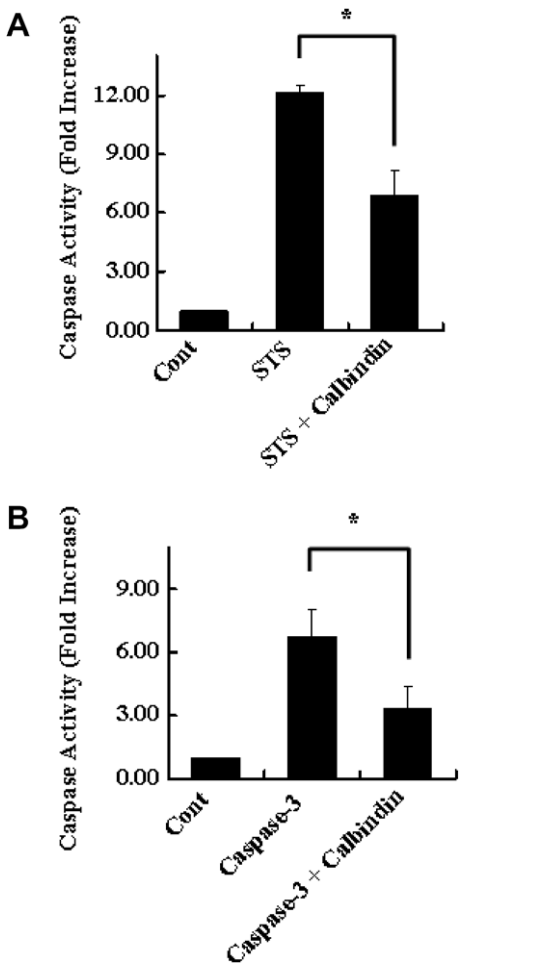

Fig. 3. Calbindin-D28K directly inhibits caspase activity. (A) Fluorogenic caspase-3 activity assay performed with 2 μg purified mouse calbindin-D28K and lysates from MN9D/Neo cells exposed for 12 h to N2 media in the absence (cont) or presence of STS. (B) Activity of purified caspase-3 (5 μg) in the presence or absence of purified calbindin-D28K (2 μg). Reaction mixtures containing only fluorogenic caspase-3 substrate served as a control. Data are expressed as fold increase relative to the untreated control (cont). Experiments were repeated three times in duplicate. $^{*}p < 0.01$.

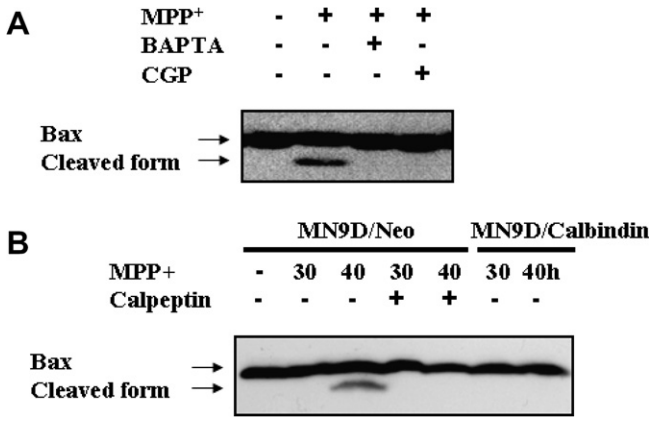

Fig. 4. Calbindin-D28K prevents calcium-mediated calpain activation during MPP^{+}-induced cell death. (A) Immunoblot analysis of calpain-mediated Bax cleavage in MN9D/Neo cells treated for 40 h with 50 μM MPP^{+} in the presence or absence of the calcium-regulating drugs, BAPTA or CGP 37157. (B) Effect of calpeptin or calbindin-D28K overexpression (MN9D/Calbindin) on calpain-mediated Bax cleavage.

ter calcium [17], and calcium-dependent, calpain-related proteolytic activity is increased in postmortem midbrain tissues from these patients. Furthermore, inhibition of calpain proteolysis by chemical calpain inhibitors, or by the overexpression of the endogenous calpain inhibitor calpastatin, significantly attenuates 1-methyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of nigral dopamine neurons [18]. Several other studies have suggested that caspase-dependent apoptosis may be the primary mechanism of dopaminergic neuronal death. This hypothesis is supported by the observations of various types of caspase activation in both experimental PD models and postmortem brains from PD patients [19–21]. Therefore, the available evidence indicates that both caspase-dependent and caspase-independent mechanisms are involved in dopaminergic neuronal death.

Previous studies carried out in rats and monkeys demonstrated that high levels of calbindin-D28K are present in midbrain dopaminergic neurons. These neurons appear to be relatively spared from neurodegeneration [22,23]. In postmortem human brains, the loss of dopamine-containing neurons in the substantia nigra pars compacta is related to the calbindin-D28K levels in this region [24]. In an MPTP-induced parkinsonian model and in weaver mutant mice, dopaminergic neurons in the substantia nigra pars compacta that are immunopositive for calbindin-D28K are more resistant to cell death than neurons that do not express this protein [25,26]. Calbindin-D28K may protect cells from neurodegeneration by buffering surges in $[Ca^{2+}]_i$, consequently stabilizing $[Ca^{2+}]_i$ [27–29]. The MN9D dopaminergic neuronal cell line used in this study consists of immortalized embryonic mesencephalic dopaminergic neurons that were established by somatic fusion [30]. Our present data support the hypothesis that calbindin-D28K plays a crucial protective role in MPP^{+}-induced dopaminergic neurodegeneration, and this effect is probably mediated by its ability to chelate calcium. The ability of calbindin-D28K to prevent the calpain-mediated cleavage of Bax in MPP^{+}-treated MN9D cells may augment its neuroprotective effect. However, as yet, it is unclear whether the MPP^{+}-induced, calpain-mediated cleavage of Bax enhances cell death in this experimental model in the same way as in other cell death models [16]. A recent report suggested that calbindin-D28K prevents osteoblast death through the inhibition of caspase activity [8]. We have previously demonstrated that STS induces cell death which is morphologically and biochemically typical of caspase-dependent apoptosis in MN9D dopaminergic neuronal cells [9]. Therefore, inhibition of caspase activity by a pan-caspase inhibitor or overexpression of pro-survival members of the Bcl-2 family is able to block STS-induced apoptotic cell death [9]. Our current data demonstrated that overexpression of calbindin-D28K in the MN9D dopaminergic neuronal cell line attenuates STS-induced apoptosis by preventing caspase activation and the subsequent cleavage of PARP. We used *in vitro* and cell-based fluorogenic substrate assays to investigate the possibility that calbindin-D28K directly binds to caspase-3 during STS-induced cell death. We found that treatment with a calcium chelator (BAPTA) does not affect the STS-induced activation of caspase, and importantly, that this phenomenon occurs independently of calbindin-D28K-mediated calcium chelation. Therefore, our data were consistent with previous report [8], since calbindin-D28K does inhibit caspase activation and can subsequently block caspase-dependent apoptosis in STS-treated dopaminergic neuronal cells. In addition, we found that the overexpression of calbindin-D28K in MN9D cells attenuates the appearance of the propidium iodide-positive necrotic nuclei induced by MPP^{+}, as well as the fragmented and condensed apoptotic nuclei induced by STS (Supplemental Figure 2). Taken together, our current data potentially explain why calbindin-D28K-expressing cells are less vulnerable to neurodegeneration, regardless of caspase or calpain activation in experimental PD models and human postmortem PD brains.

The exact role of calbindin-D28K is still not clearly understood, and this is not entirely surprising, given the complicated and diverse roles that calcium plays in both cell survival and death pathways in a wide variety of neurodegenerative models. The further identification of the molecular targets of PD-associated death signaling pathways is required in order to elucidate the function of calbindin-D28K in dopaminergic neuronal cell death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.020.

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