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# The Role of Caspase 9 during Programmed Cell Death in Ciliary Ganglia of Chick Embryos

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## Abstract

During programmed cell death (PCD) apoptosis is controlled by many factors such as proteases. With no specific protease (s) known during PCD in the developing nervous system so far, we sought to determine if any specific protease (s) is involved in this process and therefore used different protease inhibitors during PCD (from embryonic day 6 to 10) in chick embryos. Among the inhibitors commercially available, those for the inhibition of caspase 8, caspase 9 and calpain were used from embryonic day 6 to 9. Embryos were injected daily with 10 µg of the inhibitors, killed at embryonic day 10 with their ciliary ganglia (CG) excised and neurons counted. The results in this study show that only inhibition of caspase 9 but not that of caspase 8 or calpain results in a significant increase in neuron numbers of the ciliary ganglia. These results suggest a key role for caspase 9 in the intracellular pathway of programmed cell death.

**Keywords:** Programmed cell death; Caspase; Ciliary ganglia; Chick embryos

## 1. Introduction

During development of the nervous system, there is an overproduction of neurons corrected during programmed cell death (PCD). During this period more than 50% of the neurons die, even after the final synaptic contact with the target tissues [1]. It is believed that PCD is due to the lack of different target derived neurotrophic factors required for neuron survival [2]. Programmed cell death often occurs in a distinct pattern and plays a role in morphogenesis. One good example is cell death in distinct regions of developing limb bud leading to sculpting the limb [3]. This correction resulted from apoptosis controls the number of cells and therefore sizes and functions of organs. Two different internal (mitochondrial) and external (receptor mediated) pathways are known for PCD which meet each other in a common pathway of the activation of caspases. Therefore caspases are strong proteases and breaking key proteins in cell death [4,5].

Three major classes of peripheral and central nervous system neurons in chick embryos, parasympathetic ciliary ganglia (CG), sensory DRG and spinal lumbar motoneurons, undergo substantial ontogenic apoptosis between E6-E10 [6,7]. Due to the importance of PCD in development, the mechanisms involved in this programme have largely been investigated. Functionally different caspases and proteases are known, but none of

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them has been specified to be involved in the process of PCD. Previous findings from our colleagues [8] have shown that general inhibition of caspases during the period of ontogenic neuron death (E6-E10) prevents PCD significantly in ciliary ganglia of developing chick embryos. Using the same model and looking at the same ganglia (ciliary ganglia), caspase 8, 9 and calpain were inhibited *in vivo* to determine if any of these proteases is specifically involved in PCD. According to our findings only inhibition of caspase 9 prevents PCD significantly similar to the general inhibition of caspases.

## 2. Material and Methods

Inhibitors of caspase 8 (Calbiochem; Z-LEHD-FMK), caspase 9 (Calbiochem; ZETD-FMK) and calpain (Calbiochem; Z-Val-Phe-Cho) were purchased, dissolved in DMSO and diluted in PBS. Fertilized eggs were placed in a humid 37°C incubator and injected daily (from E6-E9) with 70 µl containing 10 µg of different inhibitors in DMSO through an opening of the shell onto chorio-allantoic membrane (as described by [9]). Control eggs received equal amounts of solvent (70 µl of 2% DMSO in PBS). At E10, the embryos were killed with their ciliary ganglia dissected, fixed in Bouin's fixative for overnight and processed for paraffin embedding the day after. Eight µm sections were cut, stained with haematoxilin and eosin and neurons were counted in every 10 sections according to [9].

## 3. Results

In the developing nervous system of chick and mammals, proteases are known to induce natural neuron death. To specify a protease(s), caspase or calpain, that might be involved in PCD, function of the known proteases was inhibited by using inhibitors of caspase 8, 9 and calpain. Among the three classes of neurons, parasympathetic CG, sensory DRG and spinal lumbar motoneurons, ciliary ganglia were used as a model to examine the effects of the administered inhibitors.

Experimental values of neuron counts in CGs of E10 embryos treated with different protease inhibitors during E6-E9 have been shown in Figure 1. Neuron numbers in CGs of the embryos treated with caspase 9 inhibitor showed a significant increase (5510±258; $p<0.001$) compared to that in control embryos (3903±202). Despite a slightly increased neuron numbers of CGs from chick embryos treated with inhibitors of caspase 8 (4587±588) or calpain (4574±230), statistical analysis showed that the values are not significantly different from that of the control.

At a glance, an obvious increase in neuron numbers of CGs in chick embryos treated with caspase 9 inhibitor (Fig. 2b) could also be seen in photo-micrographs in comparison with that in control (Fig. 2a). Despite significant changes in neuron numbers of CGs in embryos treated with caspase 9 inhibitor, the morphology of CGs as oval like structure having large neurons with prominent nuclei and nucleoli, was unchanged in comparison with that in control embryos. Taken together, these data indicate that caspase 9 is specifically involved in PCD.

## 4. Discussion

It is well known that PCD plays a major role in development of the nervous system with the mechanism (s) largely unknown. Caspases (cysteine-containing, aspartate-specific proteases) play key roles in apoptotic signaling in a wide range of cell types [10,11] including different neuron populations.

The results presented in previous studies from our colleagues [8] and others [12] show that general inhibition of caspases *in ovo* inhibits PCD in ciliary ganglia and motoneurons to a great extent. Also findings from Yaginuma and colleagues [13] have shown that genetic deletion of caspases 3 and 9 in mice and *in ovo* treatments with caspase 3 and 9 inhibitors results in a delayed process of PCD in motoneurons. Although involvement of the proteases and among them caspases, is known to be essential for intracellular pathway of PCD, their large numbers and spatial overlapping distribution during development hint at the

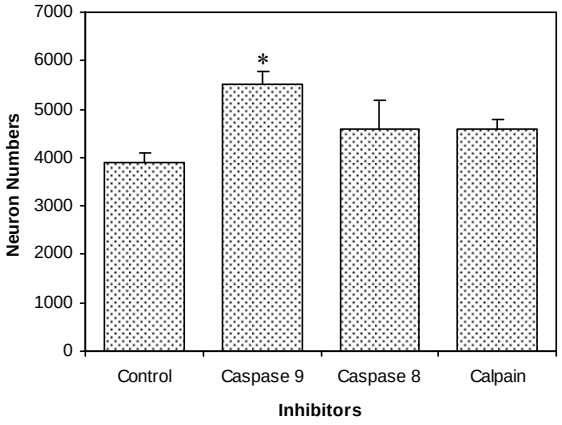


**Figure 1.** Experimental values of neuron counts in ciliary ganglia of E10 chick embryos treated with different protease inhibitors from E6-E9. While inhibition of caspase 9 significantly increases neuron numbers in comparison with that in control ($p<0.001$), inhibition of caspase 8 or calpain does not change the numbers in comparison with control ($p>0.1$).

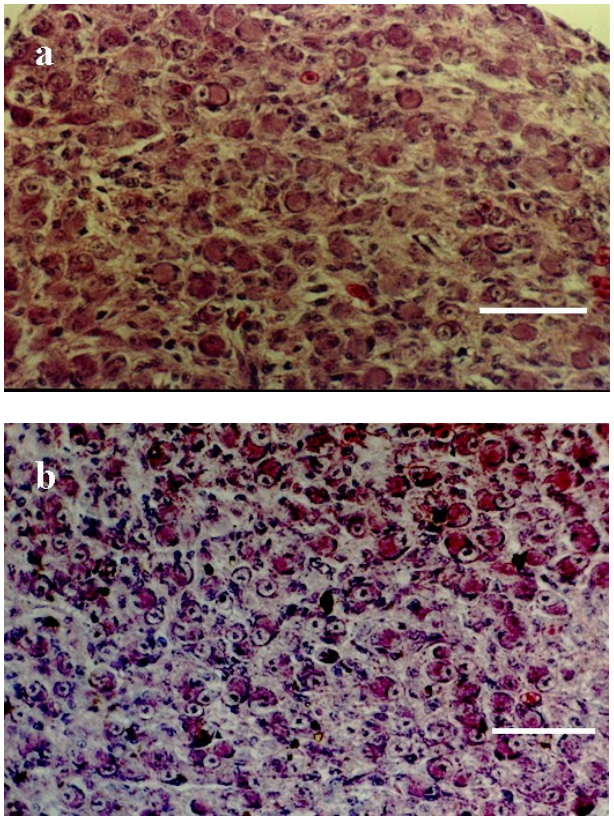


**Figure 2.** Photomicrographs of histological sections of ciliary ganglia of chick embryos treated with either DMSO diluted in PBS as a control (a) or an inhibitor of caspase 9 dissolved in DMSO and diluted in PBS (b). An increased number of neurons can easily be seen following the inhibition of caspase 9 (b) in comparison with its control (a). Scale bars represent 42 μm.

involvement of different caspases for development of different neuron populations. To specify the type of protease (s) involved during PCD, we have used inhibitors of caspase 8, 9 and calpain during PCD *in ovo* and showed that only inhibition of caspase 9 is as effective as general inhibition of caspases in preventing PCD in neurons of ciliary ganglia. Moreover, our preliminary results show that inhibiting caspase 9 prevents PCD in motoneurons as well (data not shown) to an extent reported previously by general inhibition of caspases [8,12]. To our knowledge this is the first evidence that specifies caspase 9 responsible for programmed cell death *in vivo*. Although *in vivo* studies in caspase 9-deficient mice have shown that caspase 9 is an inducer of neuron death in mouse brain and thymus [14,15], and is upregulated during development and downregulated in the adult rat brain [16], no evidence so far has been reported regarding the *in vivo* role of caspase 9 at the time of programmed cell death. There are indications however, which point to the key role of caspase 9 in the activation of other caspases such as 3, 6 and 7 [17]. For example, caspase-3 activation is abolished in caspase-9 knockout mice [15]. Also, Li and colleagues [18] have shown that there is an enhanced caspase-9 like activity in dying motoneurons. Moreover, performance of caspase 9 through two cytosolic protein factors, Apaf-1 and cytochrome *c* in mitochondria [19], which are known to be involved in neuron death, suggests that caspase 9 is the starting point for neuron death.

Although some *in vitro* evidence have pointed to the contribution of caspase 8 in the death of cultured retinal ganglion cells or cerebellar granule neurons [20,21] other studies on neuronal cultures of cerebellum, striatum and substantia nigra have denied its involvement in neuron death [22,23]. Investigating the role of caspase 8 *in vivo* during PCD, we have shown that inhibition of caspase 8 has no effect on programmed cell death in ciliary ganglia. Furthermore, our preliminary studies in motoneurons reveals the same result (data not shown), confirming that caspase 8 is not a key point of downstream signaling during PCD in different neuron populations.

In addition to the involvement of caspase family in neuron death, another family of proteases, the calpains, have also been introduced to be as effective as caspases in this process. According to a study by Villa and colleagues [24] in cultured ciliary neurons deprived trophically, inhibitors of calpains are as effective as those of caspases in preventing apoptosis and DNA fragmentation [24]. In contradiction with the results obtained *in vitro*, our results from *in ovo* studies show that inhibiting calpains during PCD does not prevent neuron apoptosis; a discrepancy that might be due to the difference between the mechanisms induce apoptosis under culture conditions and that induce apoptosis during PCD *in ovo*.

Altogether, our result is indicative of the specific role of caspase 9 in the inhibition of PCD in PNS which would also be confirmed in CNS in our future studies. Among our other perspectives is to study the *in ovo* interactive actions of caspase 9 with other proteins such as Bcl-2, Bax and Apaf known to be involved in PCD.

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