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Nicotinomid Adenin Dinucleotide Phosphate-Diaphorase (NADPH-d) Activity and CB-28 kDa Immunoreactivity in Spinal Neurons of Neonatal Rats after a Peripheral Nerve Lesion

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ABSTRACT

Our previous studies have shown that median and ulnar nerve lesion induced calbindin (CB) immunoreactivity in some injured motoneurons in developing rats. Motoneuron death induced by sciatic nerve transection in neonatal rats has been related to induction of neuronal isoform of nitric oxide synthase (nNOS). The present study investigated whether expression of CB and nicotinomid adenin dinucleotide phosphate-diaphorase (NADPH-d) activity, a marker for nNOS, is related to the death or survival of forelimb motoneurons in response to axotomy. After median and ulnar nerve transection at either P2 or P7, NADPH-d histochemistry was performed on cervical spinal cord sections to analyze the induction of nNOS in motoneurons retrogradely labeled with FB and immunostained for CB. NADPH-d reactivity was not detectable in FB labeled motoneurones up to 2 weeks after nerve lesion at P2. However, following nerve lesion at P7, some FB labeled motoneurons showed NADPH-d activity 2 weeks after nerve lesion. These NADPH-d positive motoneurons were not CB immunoreactive. The results indicate the possible role of nitric oxide (NO) in nerve regeneration and the role of CB in neuroprotection from cell death or in mechanisms of neurodegeneration. *Iran. Biomed. J. 9 (3): 103-110, 2005*

Keywords: Axotomy, Motoneuron, Nicotinomid adenin dinucleotide phosphate-diaphorase (NADPH-d), Calbindin (CB)

INTRODUCTION

Transection of peripheral nerves of rodents during the first postnatal week is known to cause death of numerous motoneurons [1-4]. Nitric oxide (NO), a neuronal messenger, has been reported to be a possible mediator of certain neuropathological states [5-7]. No is generated in the cytoplasm from L-arginine by a family of three distinct niric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). Induction of nNOS expression has shown in CNS neurons of adult mammals after facial nerve avulsion [8] and spinal root avulsion or spinal cord hemisection [9]. Also, induction of NOS has been found in neonatal but not adult motoneurons after axotomy [10], implicating NO and other free radicals are generated by the enzyme in the reaction of motoneurons to axonal injury. In our previous experiments [11] following transection of median and ulnar nerves at either P2 or P7, we showed that expression of calbindin (CB), a calcium binding protein (CBP) was induced in some injured motoneurons. There was significantly more CB immunoreactive motoneurons following lesions at P2 than P7. CB has also been found in motoneurons after sciatic nerve transection in neonatal rats [12]. CB belongs to a superfamily of structurally related CBP, which bind calcium with high affinity. It has become clear that the effects of calcium ions as important intracellular signalling molecules are closely related to CBP. CB is occured in distict subpopulation of neurons in the spinal cord, including preganglionic neurons, large neurons dorsomedial to the motoneurons, small neurons in laminae VIII and IX, and Renshaw cells. Mature spinal somatic motoneurons do not appear to express CB, although many motoneurons express CB at birth, but disappear with age [11, 13, 14].

The function of CB in injured motoneurons is not clear. To identify the possible roles of CB and nNOS in axotomized motoneurons in neonates, we

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studied nNOS expression histochemically by detecting the nicotinomid adenin dinucleotide phosphate-diaphorase (NADPH-d) reactivity, in combination with CB immunoreactivity in axotomized motoneurons, identified by retrograde transport. It has been demonstrated that distribution of NADPH-d reaction corresponds to immunocytochemical staining of neurons containing nNOS [15-17].

MATERIALS AND METHODS

Animals. Wistar rats were obtained from the Pasteur Institute of Iran (Tehran) and bred in Animal Laboratory of Shahed University (Tehran, Iran). The day of birth was counted as postnatal day zero (P0).

Surgery and tissue processing. Unilateral median and ulnar nerve cut were performed in 30 animals at postnatal days 2 (P2) and 7 (P7). Surgery was carried out under aseptic conditions. The baby rats were anesthetized by hypothermia. The median and ulnar nerves of the left forelimb were exposed to the cubital fossa and transected. Then, fast blue (FB) powder (Sigma, Germany) was applied to the proximal stumps of the cut nerves. Excess dye was removed and the skin sutured. After surgery, the rats were returned to their mother.

Animals (n = 18) were axotomized at P2 and sacrificed at P4, P7, P12, P14 and P21. Also, 18 animals were axotomized at P7 and sacrificed at P10, P12, P14, P18 and P28. In animals successfully lesioned at P2 and P7, hyperextension was observed. Although, we used the contralateral side to the lesion as control, a few rats at different postnatal ages were also used as controls. The animals were anesthetized with lethal dose of sodium pentobarbital (60 mg/kg body weight, i.p.) and perfused transcardially with saline followed by freshly prepared fixative, consisting of 4% paraformaldehyde and 0.2% picric acid dissolved in 0.1 M phosphate buffer, pH 7.4 (in some experiments picric acid was omitted). Spinal cord segments corresponding to the lower cervical (C5-T1) level were removed and post-fixed in the same fixative for 2 h before transferring to 10% sucrose in 0.1 M PBS for 1 h, and then 30% sucrose in PBS overnight. Then, 30-40 μm transverse sections from lower part of the cervical cord were cut using a cryostat. The sections were collected in 0.1 M phosphate buffer, pH 7.4 and divided into groups according to their segment of origin. Each group was processed separately throughout histochemical and immunohistochemical procedures and was known once mounted.

NADPH-d histochemistry and CB immunoreactivity. Transverse sections were first processed for NADPH-d histochemistry and then for CB immunohistochemistry. To demonstrate the NADPH-d reaction, we used the method of Vincent and Kimura [18]. Free floating sections were incubated in 20 mM phosphate buffer, pH 7.4, containing 0.3% Triton X-100, 0.25 mg/ml nitroblue tetrasolium (Sigma, Germany) and 1 mg/ml NADPH (Sigma, Germany) at 37° C for 30-60 min. The sections were rinsed in PBS, pH 7.4, and then were processed for immunostaining for CB. Sections were incubated overnight in a solution containing primary anti-CB antibody (Sigma, Germany) at a concentration of 1:2000, 0.3% triton and 3% normal serum. Fluorescent visualization of CB was achieved, as we previously described [12], by the incubation of sections with goat anti-mouse $F(ab')_2$ fragments (Sigma, Germany), conjugated with CY3, at a concentration of 1:100 for 2 h. The sections were then rinsed in PBS and mounted on gelatin coated slides. All the sections from one animal were collected on 1 or 2 slides. The slides were air-dried overnight, then rinsed in distilled water and dried again and finally were cover-slipped with Entellan. Sections were fluorescently double-labeled for CB and FB and visualized with different filter blocks on an Olympus BX50 microscope,while. Slides that were histochemically stained for NADPH-d reactivity were observed by light microscope. All the sections were examined to see if any of FB labeled motoneurons is CB immunoreactive. Cell counts were made with the nerve lesion at P7 in which some groups displayed some NADPH-d positive neurons in the FB labeled pool. By changing the filters from fluoresent to light microscopy on the same section, first all large FB labeled motoneurons and then NADPH-d positive neurons were counted.

RESULTS

In experimental animals, FB labeled motoneurons were detected in the dorsolateral cell column in the ventral horn. Labeled motoneurones showed FB in their cell bodies and proximal dendrites. Besides motoneurons, some glial cells surrounding the labeled motoneurones were also FB labeled. These glial cells are microglia and their staining result from phagocytosis of degenerating FB labeled motoneurones (Fig. 1A).

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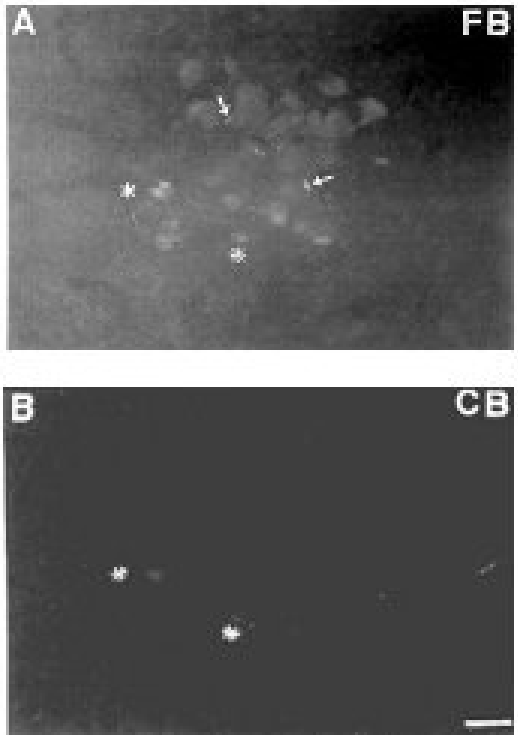

Fig. 1. Photomicrographs of A and B show a section of the cervical spinal cord 5 days after the median and ulnar nerve cut at P2. Note that injured motoneurones retrogradely labeled by FB. Some of FB labeled motoneurones are CB immunoreactive (star). Microglia are also stained by FB (arrows). Scale bar: 50 μm.

In control animals, CB immunoreactive neurons were detected with similar distribution pattern to that of seen during the normal course of postnatal development in rats [14]. Nerve cuts at P2 and P7 induced expression of CB immunoreactivity in some FB labeled motoneurones (Fig. 1B). The expression of CB in injured motoneurons was transient, as it reduced with the age of animal after the nerve cut. Table 1 shows the time course of the expression of CB in axotomized motoneurons. No CB immunoreactive motoneurons were seen contralateral to the lesion at any ages studied.

NADPH-d staining was consistently detected in all spinal cord sections of different ages. Positive neurons exhibited staining in the cell body and processes but not in the nucleus. The pattern of staining was identical at different ages and confined to the neurons in the dorsal horn laminae I-V, around central canal, and fewer neurons in the ventral horn (Fig. 2). In sections from T1, in addition to the above described neurons, the intermediolateral cell column contained groups of intensely stained neurons that linked to the neurons located around the central canal by bundles of their cell processes (Fig. 3). Positively, stained neurons exhibited different intensities of the reaction. Neurons heavily stained for NADPH-d reactivity were observed predominantly in superficial layers of the dorsal horn and around the central canal. Other neurons such as those in laminae VI and VII were moderately stained and few neurons were only weakly stained.

In experiments with a nerve cut at P2, there was no evidence of either more NADPH-d positive neurons on the injured sample in comparison to the normal smple, or any difference between sections from control and experimental animals.

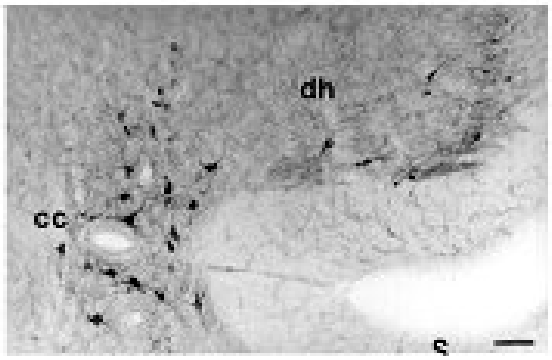

Fig. 2. This photomicrograph shows a section of lower cervical spinal cord stained histochemically for NADPH-d reaction. NADPH-d positive neurons are located in dorsal horn (dh) and around the central canal (cc). Scale bar: 100 μm.

Table 1. CB immunoreactivity in motoneurons at different postnatal ages after the nerve lesion at P2 or P7.

Age at the time of nerve lesion	Age after the nerve lesion (CB immunoreactivity)						
	P4	**P7**	**P10**	**P12**	**P14**	**P18**	**P21**
P2	+ - (n:4)	+ (n:3)	+ (n:2)	+ (n:2)	+ (n:2)	- (n:2)	- (n:3)
P7	(n:0)	(n:0)	+!- (n:4)	+ (n:3)	+ (n:4)	+!- (n:4)	- (n:3)

N, the number of rats at each group; + and - , CB positive and negative, respectively.

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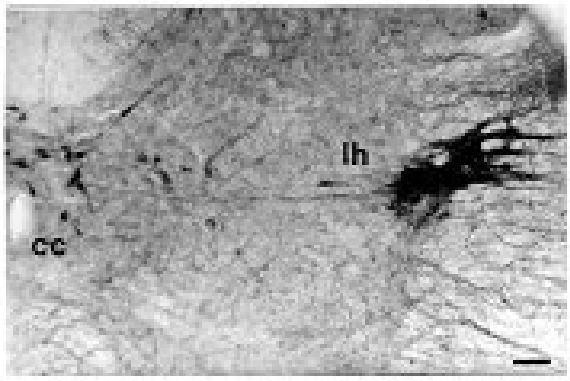

Fig. 3. A section of spinal cord at T1 level with NADPH-d staining that shows reactive neurons in the lateral horn (l h) that extends their processes towards neurons around the central canal (cc). Scale bar: 100 μm.

In these experiments, FB labeled injured motoneurons showed no NADPH-d reactivity, although some of them expressed CB immunoreactivity. So, there was no correspondence between FB labeled motoneurons and NADPH-d positive neurons in animals with a nerve cut at P2.

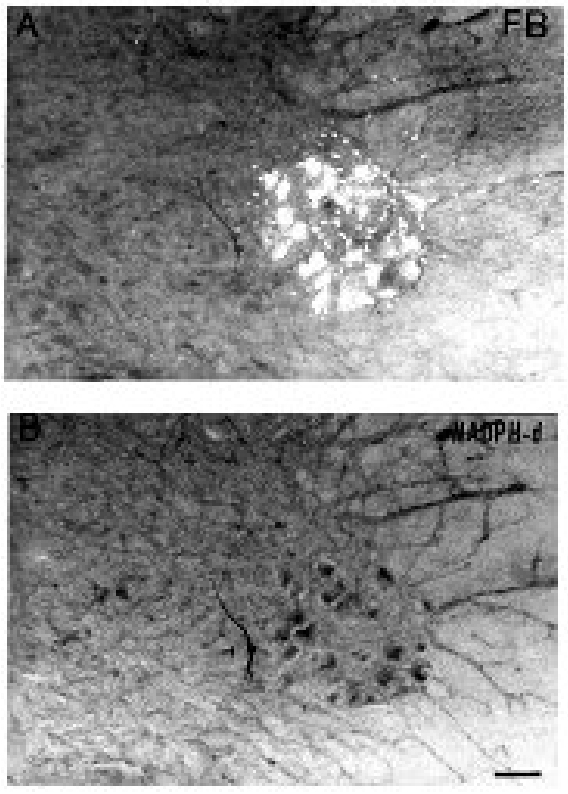

Fig. 4. A cross section of cervical spinal cord two weeks after the nerve cut at P7. Some FB labeled motoneurones show NADPH-d reactivity. A neuron intensely stained for NADPH-d reaction is seen in proximity of motor pool (arrow). Scale bar: 100 μm.

We also examined thin sections with 30 μm thickness, but nothing changed. We found some neurons with intense NADPH-d reactivity in proximity of FB labeled motoneurones (Figs. 4 and 5).

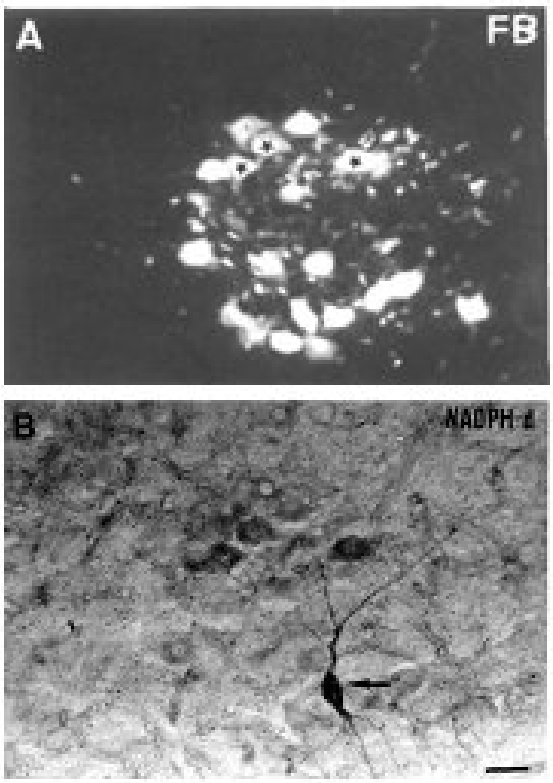

Fig. 5. A section of cervical spinal cord of a rat 14 days after the nerve cut at P7. Some motoneurones double labeled for FB and NADPH-d activity (✶). FB labeled microglia and a neuron positively stained for NADPH-d reactivity but not FB (arrow) are also seen. Scale bar: 50 μm.

However, in rats in which median and ulnar nerves were transected at P7, no NADPH-d induction was detected in FB labeled motoneurons 3, 5, 7 or 11 days after axotomy (ages; P10, P12, P14 and P18). Interestingly, from the second week onwards, some injured motoneurons weakly stained for NADPH-d reactivity and showed increased staining intensities with age, while they were not CB immunoreactive (Figs. 4 and 5). The expression of CB in some axotomized motoneurones started a week after the nerve cut at P2 or P7, but it was hardly detectable after two weeks. Therefore, only the axotomized motoneurons at P7, which detect NADPH-d reactivity were counted and the results summarized in Table 2.

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Table 2. Number of total FB labeled and NADPH-d positive motoneurons and the percentage of FB+NADPH-d$^+$/FB motoneurons in each experiment at different age groups.

Age with the nerve cut at P7	Number of FB$^+$ motoneurones	Number of NADPH-d$^+$ + FB$^+$ motoneurones	% NADPH-d$^+$+ FB$^+$/ FB$^+$ motoneurones
P10	270.0	-	-
	153.0	-	-
	244.0	-	-
	219.0	-	-
Mean (SD)	221.5 (25)	-	-
P12	245	1	0.4
	290	2	0.6
	182	-	-
Mean (SD)	239 (31.3)	1 (0.5)	0.3 (0.2)
P14	280.0	41.0	14.60
	264.0	55.0	20.80
	295.0	31.0	10.50
	251.0	28.0	11.15
Mean (SD)	272.5 (9.5)	38.7 (6.0)	14.20 (2.3)
P18	236.0	45	19.0
	255.0	33	12.9
	238.0	42	17.6
	278.0	52	18.7
Mean (SD)	251.7 (9.7)	43 (3.9)	17.0 (1.4)
P21	324	68	20.9
	355	54	15.2
	374	58	12.2
Mean (SD)	351 (14.5)	66 (4.1)	16.1 (1.7)

Regarding the difficulty in providing serial sections by cryostat in younger animals, because of missing some sections, the number of sections in younger rats was less than the older ones. Therefore, the number of total FB labeled motoneurons at different survival times, shown in Table 2, is not real. However, as can be seen in this Table, the percentage of NADPH-d positive and FB labeled motoneurons to total FB labeled motoneurons had no significant difference from two weeks onwards after the nerve cut.

DISCUSSION

The present study demonstrates the transient expression of CB in some axotomised motoneurons at P2 and P3. The aim of this study was to find any co-localisation of NOS and CB in injured motoneurons. However, we couldn not find any axotomized motoneurons expressing NOS with a median and ulnar nerve lesion at P2.

This is in disagreement with the findings of Clowry [10]. He reported that, sciatic nerve transection at P1 increased the proportion of NADPH-d positive motoneurons in dorsomedial region of the ventral horn of the lumbar enlargement between 4 and 18 days postaxotomy with a peak on the day five. This could be due to the difference in the lesioned nerve. However, it is not the case when the same experiments were performed on sciatic nerve at P2, no motoneuron showed NADPH-d reactivity. However, Clowry [10] indicated the existence of NADPH-d reactive neurons in the motor pool, while they were not FB labeled, because of the covering FB by histochemical reaction. We also found such neurons with intense NADPH-d reactivity in proximity of FB labeled motoneurones (Figs. 4 and 5). However our results correspond with the observations of some researchers [19, 20], as they didn't find the expression of nNOS enzyme by immuno-cytochemistry or its mRNA in newborn rat lumbar motoneurons following sciatic nerve transection at P2 or P3. However, peripheral nerve avulsion in adult animals [8, 9] resulted in the expression of NOS in damaged motoneurons, which is due to the difference in the type of nerve lesion.

In this study, the expression of nNOS by some motoneurons with a nerve cut at P7 may relate to

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their survival. Also, Mariotti *et al.* [21] reported that, following facial nerve cut at birth, injured motoneurones fail to show NADPH-d reactivity one or two days after the nerve cut when apoptotic changes in these motoneurones were clearly obvious. Therefore, they suggested the expression of nNOS is started after the beginning of motoneurone death.

The ability of motoneurons to survive axotomy varies with the age of animal at the time of axotomy. Pollin *et al.* [2] demonstrated median and ulnar nerve cuts in the first four postnatal days caused 80-90% of the motoneurons death and 50% of motoneurons died when the animals were one week old at the time of axotomy. The reduction in the proportion of cell death during axotomy at P7 results from increasing resistance of motoneurones to axotomy with age because they become mature. The expression of nNOS by some of the injured motoneurones could be a protective role of this enzyme in survived axotomized motoneurons, since the intensity of staining increases around the time of nerve regeneration.

The role of NO has been studied in the wallerian degeneration following sciatic nerve ligature. Gonzalez and Rustioni [22] reported that two days after the legature, nNOS appears in neurons of dorsal root ganglion, while spreads to growing fibers and reach the peak by two weeks. Also, endothelial isoform of NOS, and iNOS in macrophages increased in the site of nerve lesion. Keilhoff *et al.* [23-25] reported that, nNOS is an essential factor in peripheral nerve regeneration as sciatic nerve cut in nNOS knock-out mice caused a delay in regeneration, a substantial apoptotic cell death of spinal and dorsal root ganglionic neurons. Thus, NO supply turned out to be essential for cell survival and recovery with reference to the neuronal NOS isoform. Also, in axotomized and regenerating ganglionic neurons, NOS inhibitor reduces the inducible effect of brain derived neurotrophic factor in the expression of mRNA of growth related genes such as TAG1, L1, and GAP43 [26]. Therefore, it can be suggested that NO may involve in nerve regenerating mechanisms. However, it should be noted that growth factors might need to participate in nerve repair to bring about full recovery from axotomy at this age.

The possible role of CB in motoneurons remains unknown, as the expression of CB in injured motoneurons reduces with increasing age at the time of axotomy, so that after two weeks, its expression is hardly recognizable in injured motoneurons. Therefore, it is not surprising if there was not any co-localization of CB expression and NADPH-d reactivity in FB labeled motoneurones.

Lim *et al.* [12] reported in rats, the transient expression of CB in degenerating motoneurones of lumbar cord three days after rhizotomy of spinal nerve at postnatal day 3. They suggested, regarding the death of about %80 of injured motoneurons, the temporal expression of CB could be an unsuccessful effort of these destined to death motoneurons in buffering toxic effects of increased intracellular calcium. Another possibility is that, this protein may be involved in Ca^{2+} signaling pathways directing cell death. In our studies we demonstrated that the expression of CB in axotomized motoneurons decreases when axotomy is performed at older ages, so that in adult animals, injured motoneurons rarely express CB, as it is generally accepted that more mature motoneurons are less vulnerable to axotomy. However there are some discrepancies in the amount of cell death related to the time of axotomy in different species, even among one species. From studies in rats, some researchers reported that sciatic nerve lesion at P7 causes %80 reduction in the number of motoneurons [27], although other researchers [28] couldn't find significant reduction of motoneurones, axotomized at 5 or 6 days after birth, but they reported reinnervation of the muscle. In addition, different nuclei may respond differentially to injuries, as Clowry *et al.* [29] reported in mutant mice wobbler, motoneurones of trigeminal, abducens and hypoglossal nuclei show different responses during the motoneurone degeneration phase.

NO, in addition to its role in cytotoxicity, can involve in regenerating mechanisms. The expression of NADPH-d reaction in motoneurons axotomized at P7 but not at P2 can explain the less vulnerability of more mature neurons and their relevance to regeneration. On the other hand, temporal expression of CB in axotomized motoneurons up to two weeks after the nerve cut could be related to the primary neuroprotective response of neurons to the lethal effect of increased intracellular Ca^{2+}, or the result of involvement of this protein in cell death mechanisms mediating Ca^{2+}.

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