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Identifying and monitoring neurons that undergo metamorphosis-regulated cell death (metamorphoptosis) by a neuron-specific Caspase sensor (Casor) in *Drosophila melanogaster*

Gyunghee Lee¹, Jaeman Kim², Yujin Kim³, Siuk Yoo³, and Jae H. Park^{1,†}

¹Laboratory of Neurogenetics, Department of Biochemistry and Cellular and Molecular Biology and NeuroNet Research Center, University of Tennessee, Knoxville, TN 37996, U.S.A

²Department of Bio Sciences, Mokpo National University, Jeonnam, Republic of Korea

³Department of Life Sciences, Yeungnam University, Gyeongsan, Gyeongbuk 38541, Republic of Korea

Abstract

Activation of caspases is an essential step toward initiating apoptotic cell death. During metamorphosis of *Drosophila melanogaster*, many larval neurons are programmed for elimination to establish an adult central nervous system (CNS) as well as peripheral nervous system (PNS). However, their neuronal functions have remained mostly unknown due to the lack of proper tools to identify them. To obtain detailed information about the neurochemical phenotypes of the doomed larval neurons and their timing of death, we generated a new GFP-based caspase sensor (Casor) that is designed to change its subcellular position from the cell membrane to the nucleus following proteolytic cleavage by active caspases. Ectopic expression of Casor in vCrz and bursicon, two different peptidergic neuronal groups that had been well-characterized for their metamorphic PCD, showed clear nuclear translocation of Casor in a caspase-dependent manner before their death. We found similar events in some cholinergic neurons from both CNS and PNS. Moreover, Casor also reported significant caspase activities in the ventral and dorsal common excitatory larval motoneurons shortly after puparium formation. These motoneurons were previously unknown for their apoptotic fate. Unlike the events seen in the neurons, expression of Casor in non-neuronal cell types, such as glial cells and S2 cells, resulted in the formation of cytoplasmic aggregates, preventing its use as a caspase sensor in these cell types. Nonetheless, our results support Casor as a valuable molecular tool not only for identifying novel groups of neurons that become caspase-active during metamorphosis but also for monitoring developmental timing and cytological changes within the dying neurons.

INTRODUCTION

Metamorphosis is one of the fascinating biological events in animals as it is the critical developmental phase allowing maturational transition from a full-grown juvenile to a

[†]Corresponding author: Ph: 865-974-3035, jhpark@utk.edu.

reproductively competent adult. In insects, including fruit flies, that undergo complete metamorphosis (a.k.a. holometabolous insects), a full-grown larva transforms into a completely different looking and behaving adult. Such dramatic developmental reformation is orchestrated by the timely production of ecdysteroid hormone, which induces complete degeneration of most larval tissues, followed by *de novo* generation of adult tissues from dormant stem cells [1]. The central nervous system (CNS) is distinct from other larval tissues as the larval CNS does not undergo total destruction, but is re-developed during metamorphosis. Many larval neurons are persistently present into adulthood, but often change their anatomical structures and functions, while other neurons are programmed to die. Furthermore, the number of neurons increases by 10-fold through proliferation of post-embryonic neuroblasts during pupal development and these adult-specific neurons form elaborate and sophisticated neural circuitry to support complex adult life style [2]. Extensive CNS remodeling is also shown during the mammalian adolescent period [3–5]. While mammals are not thought to undergo true metamorphosis, significant remodeling of the nervous system during adolescence suggests parallel mechanisms at the cellular and molecular levels between insects and mammals. *Drosophila melanogaster* in particular has been a favorable subject to study CNS metamorphosis due to the advanced genetic and transgenic resources, and database [6, 7].

One of the major cellular events of CNS metamorphosis is programmed cell death (PCD) of larval neurons that will no longer be necessary in ensuing life stages [8–10]. Previously, a time-lapse TUNEL assay suggested that more than 300 larval cells primarily localized in the ventral nerve cord (VNC) undergo PCD shortly after puparium formation (APF) in *Drosophila* [11, 12]. Although neurochemical identities and biological roles of such dying cells are largely unknown, a few studies have characterized the doomed neurons. These include sixteen interneurons producing corazonin in the VNC (vCrz neurons) that are removed completely by 6–7 h APF [11–13]. In contrast to vCrz, a subset of RP2 motoneurons in the VNC degenerates at 15–20 h APF [14], while other neurons persist throughout metamorphosis, but die shortly after adult emergence (AE). For example, a majority of type-II neurons that produce high levels of the ecdysone receptor A-isoform die within a day after adult eclosion [15]. A cluster of peptidergic neurons co-producing CCAP and bursicon neuropeptide, and a pair of protocerebral neurons producing PDF neuropeptide are also eliminated approximately at the same time as are the type-II neurons [16–18]. A subset of ddaE sensory neurons persist even longer than the type-II and bursicon neurons, dying about a week AE [19]. Together, these studies indicate diversity in timing and apoptotic signaling mechanisms that induce PCD of larval neurons during metamorphosis.

Because activation of caspases is a key step that initiates apoptotic cell death, various tools have been developed to detect caspase activities. Several synthetic caspase substrates, such as Ac-DEVD-pNA, have been used to measure caspase activities biochemically [20]. Alternatively, an antibody against cleaved caspase-3 was employed to detect cells having active caspases immunologically [e.g., 21]. In addition, genetically engineered protein substrates for active caspases have been invented [22, 23]. These are useful to detect caspase-active cells selectively in a tissue that consists of a variety of cell types, because expression of them can be controlled by cell-type specific drivers. However, due to time-consuming procedures needed to detect the cleaved substrates [22] or unclear results

produced in the neurons we tested [23], we generated a new GFP-based Caspase sensor (for short, Casor). This engineered protein contains a caspase cleavage site sandwiched by two different subcellular localization signals. After processing by active caspases it changes its subcellular location from the plasma membrane to the nucleus and such molecular migration is readily detectable with a fluorescence microscope. In this study, we show that not only does Casor faithfully translocate into the nucleus once cut by activated caspases in various types of doomed neurons but it also reports developmental timing of death and progression of cytological events prior to cellular degeneration. We also discovered previously unidentified groups of cholinergic and motoneurons that are likely to die during early phase of metamorphosis.

MATERIALS AND METHODS

Casor construction

Casor was generated by adding a membrane targeted mCD8-PARP fusion fragment to the N-terminus of a nuclear targeted GFP (nGFP). The nGFP coding sequence, which contains a nuclear localization signal (NLS) in the C-terminus, was isolated from the pStinger plasmid [24] with *EcoR* I and *Spe* I digestion and then inserted into pBluescript KSII vector. This recombinant vector was digested with *EcoR* I and *Nco* I and then ligated with *mCD8-PARP* sequence that was derived from *UAS-mCD8-PARP-Venus* [22]. The resulting *pBS-mCD8-PARP-nGFP* is referred to as *pBS-Casor*. Subsequently, the *Casor* fragment (*i.e.*, *mCD8-PARP-GFP-NLS*) was inserted into *pUAST* vector at *EcoR* I and *Xba* I to generate *UAS-Casor*. Transgenic *UAS-Casor* lines were obtained via conventional germ-line transformation; a 2nd chromosome insertion line was used in this study.

Fly stocks

To drive Casor expression in specific cell types, the *UAS-Casor* flies were crossed to the following *Gal4* drivers: *Crz-Gal4* [25] and *burs-Gal4* [18] for the expression in corazonin and bursicon peptidergic neurons, respectively; *ChAT-Gal4* (Bloomington stock no. 56500) for pan-cholinergic neurons; *repo-Gal4* (Bloomington stock no. 7415), *myoglianin* (*myo*)-*Gal4* [26], and *Eaat-Gal4* [27] for glial cells; *ShakB-Gal4* (Bloomington stock no. 51633) for two common excitatory motoneuronal groups [28]. A *UAS-p35* was used to block caspase-mediated apoptosis [e.g., ref. 29].

Detection of Casor

To monitor fluorescent Casor signals in the CNS during the early course of metamorphosis, white prepupae were collected and aged at 25°C. To detect Casor in bursicon neurons, eclosed flies were collected and aged at 25°C. CNSs dissected from various metamorphic stages were fixed for 1-2 h in 1X PBS containing 4% paraformaldehyde and mounted in a quenching medium containing 80% glycerol, 0.1 M sodium phosphate buffer (pH 7.4), and 2% n-propyl galate. The images were acquired by Olympus BX61 or Keyence BZ-X710 microscope. In some experiments, cleaved Casor and Crz peptide were detected by immunostaining with anti-cleaved PARP (anti-cPARP) and anti-Crz, respectively, as described previously [11]. The primary antibodies were detected by TRITC-conjugated secondary antibodies (1:200, Jackson ImmunoResearch). Tissues were mounted and viewed

as described above. For the detection of cPARP in cultured Schneider 2 (S2) cells, the cells were fixed with 1X PBS containing 4% paraformaldehyde and then treated with anti-cPARP for 1 h. After counter staining with 0.5 µg/mL DAPI for 3 min, the cells were examined under a fluorescence microscope.

Cell transfection and UV irradiation

To express Casor in cultured S2 cells, a *pPacPL-Casor* recombinant plasmid was synthesized, where the *Casor* sequence isolated from *pBS-Casor* with *Kpn* I and *Not* I digestion was cloned into *pPacPL* vector carrying *Actin5C* promoter. S2 cells were grown at 25°C in Schneider's medium (Welgene) containing 5% fetal bovine serum (Hyclone) and 100 units/mL penicillin–streptomycin. Transfection of the *pPacPL-Casor* was carried out using FugeneHD (Promega) in a 6-well tissue culture plate according to the manufacturer's instruction. Briefly, S2 cells were seeded at a density of 1×10^6 cells/well. When cells reached 80% confluence, they were treated with 100 µL solution containing 1 µg *pPacPL-Casor* and 2.5 µL FugeneHD. Following 24 h incubation, the transfected cells were irradiated 2–3 J/cm² using UV Stratalinker™ 2400 (Spectronics), and then incubated until Trypan Blue staining or anti-cPARP immunostaining was performed.

RESULTS

Generation of UAS-Casor

Two prior studies described caspase reporters [22, 23]; however, they did not perform reliably in neurons we tested, or required a lengthy immunological procedure. In addition, it was difficult to determine positive signals unambiguously when *Gal4* drivers that are broadly expressed in the CNS were employed. These drawbacks prompted us to design a new GFP-based caspase sensor (UAS-Casor) that is readily cleaved by active caspases, leading to a change in subcellular localization. In the absence of active caspases, Casor is targeted to the membranes of the neuronal somata and their projections due to the mCD8 *trans*-membrane domain (Fig. 1). When membrane-tethered Casor is cut by activated caspases at PARP (one of the best-known caspase substrates [30]), it loses its mCD8 signal, allowing nuclear entry of the GFP with the aid of NLS. Therefore, the presence of nuclear GFP (nGFP) is an indicative of active caspases, enabling us to identify caspase-active neurons in both fixed tissues and live animals. Henceforth the intact membrane-tethered Casor is referred to as mCasor, and the caspase-cleaved product as nCasor for its nuclear localization. The nCasor can also be labeled by anti-cPARP that verifies caspase-dependent production of it. In addition, a single fluorescent signal of Casor (*i.e.*, GFP) permits conveniently co-labeling of nCasor-positive neurons with different fluorophores, which can provide valuable information on their neurochemical identities.

Nuclear translocation of Casor in doomed vCrz neurons

To test if Casor works as we desired, it was expressed in a set of peptidergic neurons that produce the neuropeptide Corazonin (Crz). In a larval CNS, Crz is produced from eight bilateral pairs of neurons (vCrz) that locate from 2nd thoracic to 6th abdominal neuromeres in the VNC, from three neuronal pairs in the dorso-lateral (DL) protocerebrum, and from one pair in the dorso-medial (DM) protocerebrum [17, 31]. These three groups of neurons

have distinct developmental fates; the DL neurons survive into adulthood, the DM neurons survive up to 24–48 h APF, and the vCrz neurons degenerate progressively over 6–7 h APF in a caspase-dependent manner [11–13, 17].

Because the vCrz neurons are well characterized in regard to apoptotic fate, we first tested how Casor behaves in these neurons. During larval stages and at 0 h APF, mCasor signals readily labeled intricate axonal processes and somata of the vCrz neurons (Fig. 2a, ai). Since cytological signs of the vCrz degeneration was first observed at 1–2 h APF [11], we anticipated that caspase activation takes place around this metamorphic time. Consistent with this prediction, we found GFP signals almost exclusively within the nuclei of all sixteen vCrz neurons at 2 h APF (Fig. 2b, bi), indicating that activated caspases cleaved mCasors successfully. The expression of Casor did not interfere with normal course of vCrz PCD as all GFP signals disappeared by 7 h APF (Fig. 2c).

To further corroborate the cleavage of Casor by the activated caspases, a caspase inhibitor p35 was co-expressed in the vCrz neurons. As expected from our previous studies [11, 13], vCrz PCD was blocked by p35 and mCasor signals remained intact even at 7 h APF (Fig. 2d, di). These results indicate that nuclear translocation of Casor is indeed due to its proteolytic cleavage by activated caspases.

Time-lapse caspase activation in vCrz neurons

To determine the metamorphic timing of caspase activation more precisely, nCasor signals were tracked as soon as a larva turns into white puparium. At around 30 min APF, we began to detect weak but clear nCasor signals in an average of six neurons in a stochastic manner (Fig. 3a vs. b; Table 1). The signals were also visible in a few neurons in a live prepupa as early as 10 min APF (Supplemental Fig. 1). These results indicate that caspase activation in some vCrz neurons is nearly coincident with the onset of metamorphosis.

Around 1 h APF, more vCrz neurons became nCasor-positive and the nuclear fluorescent signals significantly intensified. The enhanced nuclear signals most likely reflect increased accumulation of nCasors within a smaller nucleoplasm (Fig. 3c). By 1.5–2 h APF, Casor signals were found almost exclusively in all vCrz nuclei (Fig. 3d, e; Table 1). However, axons are still largely intact at this stage, as revealed by anti-Crz at 2 h APF (Fig. 3f). The data suggest a broad distribution of active caspases throughout the somata and axons of doomed neurons, and that the nuclear importing machinery is unaffected by caspases in early apoptotic neurons.

Progressive apoptotic events in bursicon neurons

In contrast to the timing of vCrz neuronal death, a group of 14–16 peptidergic neurons located in the dorsal surface of the VNC are programmed to die within 24 h AE [16, 18]. These neurons express both CCAP and bursicon (burs) neuropeptides, thus we previously referred to them as bursCCAP neurons [18]. Since we labeled these neurons by using a *burs-Gal4* driver in this study, we will hereafter simply designate them as burs neurons. Their large-sized somata and positions close to the dorsal surface facilitate convenient microscopic observations.

Our previous time-course studies revealed that about 25% of burs neurons are eliminated by 8-9 h AE [18]. These results imply that caspase activation occurs earlier than this post-eclosion time. To determine the developmental timeline of caspase activation, Casor expression driven by the *burs-Gal4* was monitored between 0 and 6 h AE. In newly emerged adult flies (0 h AE), only mCasor signals were detected (Fig. 4a, ai). The nCasor was first observed in a few neurons from the samples dissected at 1 h AE (arrowheads in Fig. 4b, bi; Table 2). During the following 2 hours, nCasor-positive neurons increased rapidly in number (Fig. 4c–e, ci; Table 2). By 3–4 h AE, all burs neurons showed intense nCasor signals and their somata were also noticeably shrunken (Fig. 4e, f; Table 2).

Unexpectedly but interestingly, we noticed nuclear-sequestered nCasor signals dispersed back to the cytoplasm in some of burs neurons around 5 h AE (arrowheads in Fig. 4g, gi, n=7). By 6 h AE, more neurons showed such diffused pattern (Fig. 4h, hi, n=12). The most likely cause of the loss of nCasor is the disintegration of the nuclear envelope as a result from the actions of active caspases. These results together demonstrate that Casor is very useful not only for determining the metamorphic timing of caspase activation in dying neurons, but also for monitoring dynamic cellular events associated with the caspase action.

Apoptosis of motoneurons

Using Casor, we extended our studies to identify larval motoneurons that might undergo PCD during prepupal development. *ShakB-Gal4* labels two distinct types of the motoneurons [28, 32]. There are 10 pairs of Common Excitatory dorsal motoneurons (CEd) located from 1st thoracic (T1) to 7th abdominal (A7) neuromeres (Fig. 5a, c). The abdominal CEd neurons were shown to be a larval version of embryonic RP2 motoneurons [28]. Another group consists of 11 pairs of Common Excitatory ventral motoneurons (CEv) located from T1 to A8 neuromeres (Fig. 5d, f). Both types of motoneurons are glutamatergic and innervate longitudinal muscles on the dorsal and ventral body wall, respectively [28].

The CEd neurons positioned in A2–A7 segments were previously shown to undergo PCD during 15–20 h APF [14]. In contrast to the A2–A7 CEd neurons, we found two nCasor-positive CEd pairs in T2 and T3 segments at 2 h APF (arrowheads in Fig. 5b). The nuclear entry seemed slightly delayed in the T2 neurons, implying that initiation of caspase activation is not completely synchronized between T2 and T3 neurons. Nevertheless, the results suggest segment-specific apoptosis among CEd neurons. The segment-specific apoptotic fate of motoneurons during metamorphosis is also described in the moth *Manduca sexta* [33], suggesting that differential signaling mechanisms must operate to avoid untimely death of motoneurons in each segment.

In comparison to CEd, we found all CEv neurons displaying nCasor expression at 2 h APF (Fig. 5e). To verify the caspase activities in these neurons, immunostaining with anti-cPARP was performed. As shown, nCasor signals in the CEv neurons overlapped with cPARP-immunoreactivity (Fig. 5g–i), supporting that nCasor is a product of active caspases. Taking these data together suggests that *ShakB*-positive thoracic CEd and all CEv motoneurons are programmed to die approximately at the same metamorphic stage as are vCrz peptidergic neurons.

Identification of doomed cholinergic neurons in the CNS

Cholinergic neurons are widely distributed in the larval CNS. ChAT (Choline Acetyl Transferase) is an essential enzyme required for acetylcholine biosynthesis, and thus serves as a histological marker for all cholinergic neurons [34]. Indeed *ChAT-Gal4* (a.k.a. *Cha-Gal4*) driven Casor expression patterns are quite broad throughout the CNS. At 2-3 h APF, we detected some of the cholinergic neurons that are positive for nCasor (Fig. 6, n=5). However, it is challenging to clearly delineate nCasor signals from intense mCasor background stemming from dominant non-apoptotic cholinergic neurons. To resolve this issue, immunolabeling of the nCasor with anti-cPARP was performed, because cPARP-immunoreactivity (IR) does not produce mCasor-like background. The results showed overlapping of cPARP-IR and nCasor signals (arrowheads in Fig. 6), suggesting that some larval cholinergic neurons are programmed toward apoptotic death.

Caspases in the larval sensory neurons

Metamorphic apoptosis of larval sensory neurons has not been well investigated, except for a subset of dendritic arborization (da) neurons [19, 35]. Since all larval sensory neurons in the PNS are known to be cholinergic [34], we employed *ChAT-Gal4* and *UAS-Casor* to identify any of these neurons undergoing apoptosis during early metamorphosis. Around 3-4 h APF, a few neurons at various locations in the thoracic epidermis were found to carry nCasor expression, and such signals were also positive for cPARP-IR (arrowheads in Fig. 7a-c). However, it is too premature to tell if these nCasor-positive neurons undergo apoptotic death. It is possible that these neurons do not die but remodel via neurite pruning that might involve locally active caspases, as described for the dendritic pruning of da neurons [35]. However, caspase activation takes place only after the severance of dendrites from the soma, and then the caspases facilitate the removal of the fragmented dendrites [22]. In such case, nuclear import from the severed neurites is unlikely to happen. In contrast, nCasor signals observed in the cholinergic neurons suggest that these neurons have intact projections as shown for vCrz neurons. Hence activation of caspases within these neurons during early metamorphosis is likely to reflect their apoptotic fate.

Behavior of Casor in non-neuronal cell types

Besides neuronal cells, glial cells are the other major cell type of the CNS. Since metamorphosis-associated glial cell death has been largely unexplored, we investigated if Casor can be used to detect PCD of glial cells. Casor expression in glial cells was performed with a pan-glial driver *repo-Gal4* [36]. As expected, expression of *mCD8GFP* by this driver showed broad signals in the larval CNS (Fig. 8a). To our surprise however, Casor expression was quite different from the mCD8GFP pattern. In contrast to the broadly diffused pattern of mCD8GFP signals, Casor produced a spotty pattern of distribution (Fig. 8b). Similar patterns were also observed using other glial drivers such as *myo-Gal4* and *Eaat1-Gal4*. To see if the punctate signals are derived from nCasor, we employed an independent nuclear marker, Red-stinger [e.g. ref. 37]. Co-expression of Casor and Red-stinger showed that Casor signals distributed mostly at nuclear periphery and did not co-localize with the Red-stinger (Fig. 8c-f). Thus we speculate that such punctate pattern of Casor signals is mostly likely due to aggregation of Casor proteins that failed to target the plasma membrane.

Of interest, a fraction of Casor signals was also positive for cPARP-IR (Fig. 9a-aii). Moreover, the cPARP-IR was absent from co-expression of p35 (Fig. 9b, bi), suggesting that the cPARP-IR was indeed a result from the cleaved Casor by caspases. It is not clearly understood why Casor failed to target the membrane but formed aggregates in the cytoplasm. Since expression of either mCD8-GFP or nGFP alone does not display aggregated patterns, we speculate that the presence of two conflicting trafficking signals, signal peptide (SP) and NLS, present in the Casor might be a culprit for aggregate formation. In line with this, it is notable that normally a nuclear protein Myc accumulates in the cytoplasm if it is fused in frame with SP [38]. Expression of a fusion protein consisting of membrane-targeted myrGFP and RFP-NLS also produced aggregates in perinuclear region [39]. These studies support that the two conflicting signals in a single protein often compromise protein sorting and trafficking in some cell types.

Next, we investigated Casor's behavior in apoptotic S2 cells in culture. For this, S2 cells were transfected transiently with *pPacPL-Casor* vector as described in Materials and Methods. The number of transfected cells and intensity of Casor signals were proportional to the plasmid concentrations. However, Casor appeared to form aggregates in the cytoplasm of normal S2 cells as observed in glia cells (Fig. 10a–c). When cell death was induced by UV-irradiation, Trypan Blue-positive cells displayed irregular shapes, a sign of death. About 50% of S2 cells died 2 h after exposure and the mortality peaked by 8 h after UV-irradiation (Fig. 10d–g). In these dying cells, cytoplasmic Casor aggregates remained without apparent nuclear entry; however, cPARP-IR was partially overlapped with Casor aggregates (Fig. 10h–j), whereas untreated S2 cells were completely negative for cPARP-IR (Fig. 10k–m). Based on these results, we speculate that cPARP-IR signals in UV-irradiated cells were generated by activated caspases, but the failure of cleaved Casor to enter the nucleus is due to physical hindrance imposed by aggregate formation.

DISCUSSION

Casor is a reliable apoptosis sensor in neuronal cells

During metamorphosis, many juvenile tissues and organs are programmed to degenerate via apoptosis and/or autophagy. We propose to use a new term, **metamorphoptosis**, to distinguish metamorphosis-regulated cell death (metamorphoptosis) from other types of cell death that are associated with such as embryonic development or tissue homeostasis. This term can be used broadly not only in insects/invertebrates, but also in vertebrates including amphibians and even mammals. In mammals, the pubertal and adolescent transformation can be considered a variation on the theme of metamorphosis with respect to the morphological, physiological, neurological and behavioral changes that lead to the physical as well as mental readiness of reproduction and independent life [40, 41]. A unique feature of the metamorphoptosis is that it is regulated primarily by endocrine hormonal signals, which orchestrate diverse metamorphic events including cell death [1, 42].

We have particularly been interested in the mechanisms of metamorphoptosis of neurons. Here we have shown that Casor is a simple but effective neuron-specific *in vivo* caspase sensor; *trans*-localization from the membrane to the nucleus after processing by caspases allows convenient identification of doomed neurons. Casor acts reliably as an apoptotic

reporter in two groups of peptidergic neurons (vCrz and burs) that have been well-characterized for metamorphoptosis. Detection of nCasor in the vCrz neurons is observed ahead of axonal degeneration that has been recognized the earliest morphological apoptotic sign. Thus, nCasor signals can be used as the earliest apoptotic marker. Casor is also faithfully nuclear-localized in some cholinergic larval neurons in both CNS and PNS that have not been characterized previously. We also discovered two types of motoneurons undergoing metamorphoptosis soon after puparium formation. They are the first identified motoneurons dying at this early phase of metamorphosis. These results demonstrated that Casor can serve as an effective apoptotic sensor for various neuronal cell types.

Another benefit of using Casor is that cPARP-IR can be employed as a complementary tool. In contrast to *Crz*-, *burs*- and *ShakB-Gal4* drivers that each directs Casor expression in a small number of neurons, *ChAT-Gal4* labels quite a large number of heterogeneous neurons varying their soma sizes, shapes, positions, functions, and fates. Thus, it was extremely challenging to distinguish cells containing nCasor from the background of non-apoptotic neuronal population. In such case, cPARP-IR is beneficial, eliminating widespread PCD-irrelevant mCasor background. With this complementary tool, Casor is a valuable transgenic reagent to detect previously unidentified dying neurons by utilizing any existing neuronal *Gal4* drivers.

A growing body of evidence shows that various cellular events require non-lethal actions of caspases [43–48]. Therefore, results from Casor or other similar reporters should be interpreted carefully. Nonetheless, Casor can serve as a sensitive marker for caspase-active neurons, which are prime candidates undergoing metamorphoptosis.

Casor reports dynamic cellular events in dying neurons

Casor provided us with valuable information on ongoing cellular events in dying neurons. First it appears that active caspases are broadly distributed throughout neuronal projections. This is supported by rapid clearance of mCasor from extensively projected axons (Fig. 3e, f). Such widespread caspases may play a key role in simultaneous and swift degeneration of entire structure of neurons. Second, nuclear-cytoplasmic trafficking machinery operates normally during early phase of neuronal apoptosis. The nuclear entry of the nCasors seems quite rapid, considering lengthy axonal projections through which the nCasors must be delivered to the nucleus. It has been shown that importin-mediated retrograde trafficking is important for the long-distance delivery of synaptically-localized proteins to the nucleus [49, 50]. These proteins undergo synaptic activity-dependent processing, resulting in the exposure of the NLS. Our data further suggest that importins are widespread throughout the axons and deliver cargos that are produced anywhere along the axons. Third, dispersion of nCasor back to cytoplasm after completion of nuclear entry indicates apoptotic disintegration of the nuclear envelope and/or the gate-keeping nuclear pore complex. It has been suggested that initial caspase activation compromises the nuclear pore complex, which then allows diffusion of effector caspases into the nucleoplasm [51]. Caspase-mediated degradation of nuclear lamins and other nuclear molecules induces subsequent disassembly of the nuclear envelopes [52]. In this respect, Casor is an excellent tool to investigate the temporal events from the beginning of caspase activation to nuclear apoptosis.

Casor can also help to estimate developmental timing of the initiation of apoptotic program. For metamorphosis of vCrz neurons, ecdysone steroid hormone is the key developmental cue. Activated ecdysone receptors act to induce transcription of *grim* cell death gene directly or indirectly, which then causes caspase activation [12]. As we reported here, caspase activation in the vCrz neurons took place almost immediately after the larvae initiate their metamorphic changes, suggesting that the ecdysone-induced *grim* expression in the vCrz neurons must have happened prior to the onset of metamorphosis. In other words, transcriptional *grim* gene induction is likely to be done in the wandering larval CNS in response to a late larval ecdysone surge [53]. In contrast to vCrz neurons, RP2 motoneurons in A2–A7 neuromeres start to die at 15 h APF in response to a small prepupal ecdysone pulse [14]. It is unknown why the death of RP2 neurons is not triggered by the earlier (late larval) ecdysone peak. Determination of timing of caspase activation using Casor in these neurons might provide interesting insights into these phenomena.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AE	after eclosion
AFP	after puparium formation
CNS	central nervous system
NLS	nuclear localization signal
PCD	programmed cell death
PNS	peripheral nervous system
SP	signal peptide
VNC	ventral nerve cord

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Fig. 1.

A diagram showing Casor structure. The 40-aa peptide derived from the human PARP1 (hPARP) contains caspase cleavage site. NLS is obtained from *Drosophila* Transformer. In the cells containing active caspases, the released nCasor (cPARP-GFP-NLS) enters the nucleus via the NLS. Cleaved PARP (cPARP) can also be detected by anti-cPARP.

Fig. 2.

Nuclear targeting of Casor in the doomed vCrz neurons. **(a, ai)** Intact Casor was detected in all somata and axonal projections at 0 h APF. **(b, bi)** nCasor signals were detected in all neurons at 2 h APF. **(c)** Lack of GFP signals indicates death of all vCrz neurons at 7 h APF. **(d, di)** Inhibition of caspases by co- expression of p35 rescued vCrz apoptosis and prevented nCasor production at 7 h APF. ai-di. Magnified images indicated in brackets of a–d. scale bar, 50 μ m

Fig. 3.

Time-lapse of caspase activation in vCrz neurons immediately after puparium formation (APF). **(a)** At 0 h APF, intact Casor labeled all somata and projections. **(b)** At 0.5 h APF, nCasor signals were detected in a few neurons as indicated by arrowheads. **(c, d)** The numbers and intensity of nCasor signals gradually increased while GFP signals in the projections became fainter. **(e)** At 2 h APF all neurons showed mostly nCasor with very faint axons. **(f)** Crz-immunoreactive signals at 2 h APF. Projections and somata were clearly visible (arrowheads). See also Table 1 for quantification. scale bar, 50 μ m

Fig. 4.

Progression of caspase activation in burs neurons shortly after adult emergence (AE). **(a)** At 0 h AE, all neurons displayed mCasor expression only. **(b)** The earliest detection of nCasor in a few neurons at 1 h AE. Arrowheads indicate strong (closed) or weak (open) nCasor signal, respectively. **(c–f)** Gradual increase in the numbers and intensity of nCasor signals at indicated time-points. See also Table 2 for quantification. **(g)** At 5 h AE, nCasor signal began to disperse back to the cytoplasm, as indicated by arrowheads. **(h)** At 6 h AE, the dispersed nCasor signals became noticeably dimmer (arrowheads). **(ai–hi)** Magnified images selected from specimen collected at indicated time-points to show representing status of Casor. scale bar, 50 μ m for a–h; 10 μ m for ai–hi

Fig. 5. PCD of CEv and CEd motoneurons. Casor was expressed in CEv and CEd larval motoneurons by *ShakB-Gal4*. **(a)** Casor expression in CEd in a larval CNS. **(b)** Casor expression in CEd at 2 h APF. White arrowheads indicate T2 and T3 CEd motoneurons containing nCasor signals. **(c)** A diagram showing 10 pairs of CEd from T1 to A7 segments. **(d)** Casor expression in CEv neurons in a larval CNS. **(e)** Casor expression in CEv at 2 h APF. All CEv neurons displayed nuclear nCasor expression. **(f)** A diagram showing 11 pairs of CEv motoneurons located from T1 to A8 segments. **(g, h)** Double labeling of nCasor

(green, g) and cPARP-IR (magenta, h) in abdominal CEv motoneurons at 2 h APF. **(i)** Merge of Casor and cPARP (white) demonstrates a complete match of the two signals. scale bar, 50 μm

Fig. 6. Caspase-active cholinergic neurons in the CNS during early phase of metamorphosis (2-3 h APF). *ChAT-Gal4* was used to drive Casor expression. nCasor signals are indicated by arrowheads. These signals are also detected by anti-cPARP. scale bar, 50 μ m

Fig. 7. Caspase activation in cholinergic sensory neurons during early metamorphosis (3-4 h APF). (a–c) The sensory neurons are shown in three different areas of the pupa. nCasor signals are indicated by arrowheads (left panels), and these signals overlap with anti-cPARP (middle and right panels). scale bar, 50 μ m

Fig. 8.

Formation of Casor aggregates in glial cells. Reporter expression was driven by a *repo-Gal4* in glial cells. **(a)** mCD8GFP. **(b)** Casor. **(c)** Redstinger (nuclear RFP). **(d–f)** An area magnified from **b**. A composite image (f) shows that Casor aggregates do not overlap with most Redstinger-labeled nuclei. scale bar, 100 μ m

Fig. 9.

Capsase activity in glial cells. **(a-aii)**. Overlapping Casor signals with cPARP-IR. **(b, bi)** Co-expression of Casor and p35 to inhibit caspases (n=12). Negative cPARP-IR (bi) supports that cPARP-IR signals shown in (ai) result from the caspase activity. scale bar, 20 μ m for a-aii, 100 μ m for b-bi.

Fig. 10. Expression of Casor in S2 cells. **(a–c)** Dosage-dependent expression of Casor. The numbers of transfected cells and intensity of Casor signals were proportional to the amounts of *pPacPL-Casor* vector used for transfection as indicated in each panel. **(d–f)** Increase of dying S2 cells was shown in 0 h **(d)**, 2 h **(e)**, and 8 h **(f)** after UV-irradiation. Trypan Blue was used to detect apoptotic cells (arrowhead in **e**). **(g)** Mortality of UV-irradiated S2 cells at indicated time-points. **(h–j)** A representative single S2 cell at 8 h after UV-irradiation. The cells were fixed and processed for the detection of Casor **(h)** and cPARP-IR **(i)**. **(j)** Merge of

(h) and (i). DAPI staining shown in blue indicates the nucleus (N). **(k–m)** Control S2 cells. They are positive for Casor (k) but negative for cPARP-IR (l). (m) Merge of k and l. scale bar, 50 μm for a–f, 5 μm for h–m.

Average of numbers of vCrz neurons displaying nCasor signals at indicated time-points. (SD, standard deviation; APF, after puparium formation; n, number of specimen)

Table 1

APF (h)	0	0.5	1	1.5	2
mean ± SD	0 ± 0	6 ± 2	8.3 ± 3.7	14.5 ± 5.1	15.8 ± 0.5
n	5	6	10	6	6

Table 2

Average numbers of burs neurons displaying nCasor signals at indicated time-points. (SD, standard deviation; AE, after eclosion; n, number of specimen)

AE (h)	0	1	2	3	4
mean $\pm$ SD	0 $\pm$ 0	1 $\pm$ 1.2	12.6 $\pm$ 1.5	15.2 $\pm$ 0.5	13 $\pm$ 2
n	10	7	8	5	5