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·**Perspective**·

Non-apoptotic role of caspase-3 in synapse refinement

Jin-Yuan Wang, Zhen-Ge Luo

Institute of Neuroscience, State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Corresponding author: Zhen-Ge Luo. E-mail: zgluo@ion.ac.cn

Caspases, a family of cysteine proteases, mediate programmed cell death during early neural development and neurodegeneration, as well as following neurotoxic insults. Notably, accumulating lines of evidence have shown non-apoptotic roles of caspases in the structural and functional plasticity of neuronal circuits under physiological conditions, such as growth-cone dynamics and axonal/dendritic pruning, as well as neuronal excitability and plasticity. Here, we summarize recent progress on the roles of caspases in synaptic refinement.

Keywords: caspases; neuronal plasticity; synaptic refinement

Since the discovery of the *Caenorhabditis elegans* caspase gene *ced3*[1], 12 mammalian caspases have been identified, including initiator caspases (caspase-1, -2, -5, -8, -9, -10, -11, and -12) and effector caspases (caspase-3, -6, -7, and -14). Among these, caspase-3 plays a critical role in mediating apoptosis in both the death receptor pathway and mitochondrial pathways[2]. In the nervous system, caspases not only mediate cell death during neural development and neurodegeneration[2], but also play non-apoptotic roles under physiological conditions, e.g., synaptic plasticity[3, 4], dendritic pruning in *Drosophila*[5, 6], and the chemotropic response of axonal growth cones[7]. Recently, we found that caspase-3 plays an important role during synapse refinement at the neuromuscular junction (NMJ)[8] (Fig. 1A). Like other synapses, the development of NMJs involves a complicated refinement process. At early-to-middle embryonic stages, myotubes form spontaneous pre-patterned acetylcholine receptor (AChR) clusters, and after the invasion of motor nerves, the innervated clusters are strengthened and stabilized, while aneural AChR clusters are gradually dispersed[9]. The interplay between positive and negative factors determines the precise matching of presynaptic nerve terminals and postsynaptic structures on the muscle surface. The motoneuron-derived glycoprotein agrin is believed to be the critical positive factor, ablation of which causes severe defects in formation of the NMJ[9]. Interestingly, the neurotransmitter acetylcholine (ACh) has been proposed to be a negative factor[10, 11]. Genetic ablation of *choline acetyltransferase* (*ChAT*) partially rescues AChR clusters in agrin-knockout mice[11] and treatment of cultured muscle cells with carbachol (CCh), a non-hydrolyzable cholinergic agonist, induces the dispersion of AChR clusters[10, 11]. Intriguingly, genetic evidence and results from cultured muscle cells suggest that Cdk5, a cytoplasmic serine/threonine kinase, is an effector in dispersing AChR clusters[11, 12]. More recently, Lee and colleagues reported that the intermediate filament protein nestin is required for ACh-induced association of p35, the co-activator of Cdk5, with the muscle membrane and Cdk5 activation[13]. Similar to the effect of Cdk5 inhibition or ablation, knockdown of nestin in agrin-deficient mice markedly rescues AChR clusters[13]. How does agrin counteract the role of ACh in Cdk5 activation? In a previous study, we showed that CCh stimulation of cultured muscle cells activates the Ca^{2+}-dependent protease calpain, leading to the cleavage of P35 to P25, a more stable and stronger activator of Cdk5[14]. Interestingly, rapsyn, a postsynaptic scaffold protein associated with AChRs, physically interacts with calpain and inhibits its activity. Agrin, by increasing the interaction between calpain and rapsyn, inhibits calpain activity[14]. We

noted that the loss of AChR clusters in agrin-mutant mice is partially rescued by injecting or over-expressing calpain inhibitors in muscle cells[14], to a much lesser extent than that found with *ChAT* ablation. This result prompted us to search for other downstream mediators in the activity-dependent elimination of AChR clusters.

Because caspase-3 is activated at the NMJ in patients and mice with slow-channel syndrome resulting from mutations in certain subunits of AChRs and a sustained elevation of Ca^{2+} concentration in muscles[15], we hypothesized that caspase-3 might be involved in synapse refinement. We found that cholinergic stimulation of cultured muscle cells activates caspase-3 locally in AChR cluster-enriched regions, and notably, active caspase-3 is associated with aneural AChR clusters[8]. Inhibition or genetic ablation of caspase-3 stabilizes AChR clusters *in vitro* and *in vivo*. In line with this notion, the decrease of apoptotic protease activating factor 1 (Apaf-1), an adaptor protein essential for caspase-3 activation in the mitochondrial pathway, also stabilizes AChR clusters. It remains of interests to determine whether and how apoptosomes are recruited to the AChR complex, and are thus involved in cluster dispersion.

We also investigated the mechanism by which caspase-3 functions in the disassembly of AChR clusters and identified Dishevelled1 (Dvl1), a Wnt signaling protein that mediates agrin/MuSK signaling in AChR clustering[16] (Fig. 1A). Blockade of Dvl1 cleavage also stabilizes AChR clusters in culture and *in vivo,* indicating that Dvl1 is a functional substrate of caspase-3[8].

Several lines of evidence support the hypothesis that agrin stabilizes synapses at least partially through counteracting the negative role of ACh during NMJ development. How does agrin limit caspase-3 activation? Interestingly, we found that heat shock protein 90β, which regulates AChR cluster formation and maintenance by stabilizing rapsyn[17], is involved in agrin signaling in restraining caspase-3 activity[8] (Fig. 1A). It has been shown that during dendritic pruning in *Drosophila*[5] and songbird learning[18], caspase-3 activity is strictly controlled by X-linked inhibitor of apoptosis protein (XIAP). Thus, caspase-3 activity is tightly controlled in various physiological conditions during processes of neural development and plasticity.

Does caspase-3 also participate in structural or functional synaptic plasticity in the central nervous

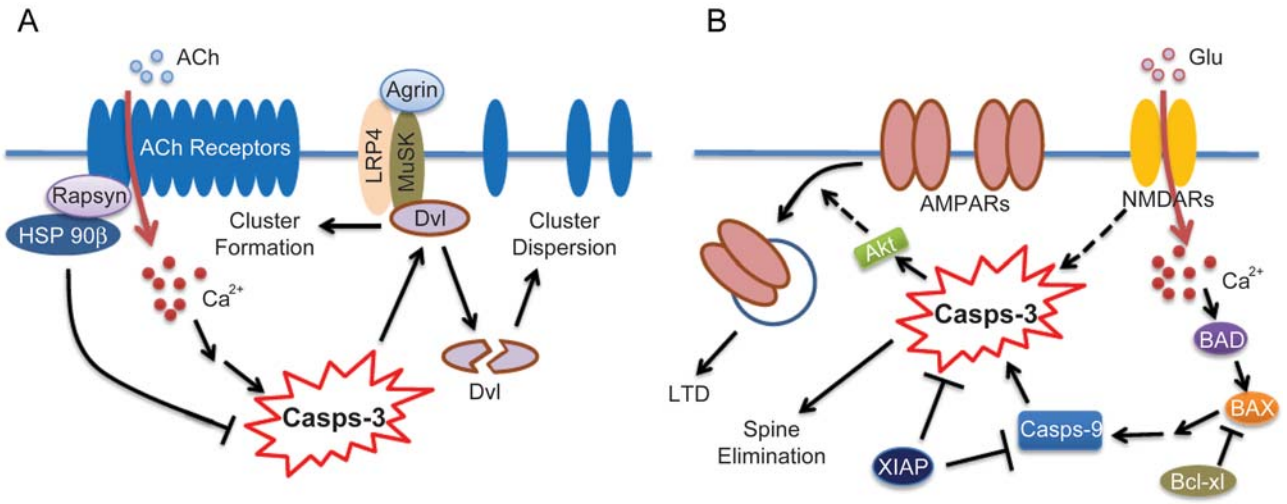

Fig. 1. Non-apoptotic role of caspase-3 in synapse elimination and plasticity. (A) At the NMJ, ACh stimulation increases the intracellular Ca^{2+} concentration, thus activating caspase-3 (casps-3) at postsynaptic sites. Active caspase-3 cleaves Dvl, an adaptor protein that mediates agrin/MuSK signaling, leading to the dispersion of aneural AChR clusters. The rapsyn-associated protein HSP 90β restricts and tightly controls caspase-3 activity at the postsynaptic regions innervated by motor neurons. (B) At the CNS excitatory synapse, NMDA receptor stimulation activates the BAD-BAX-caspase-3 cascade, which causes AMPA receptor internalization and consequently, NMDA-dependent long-term depression (LTD) and spine elimination. AKT acts as the substrate of caspase-3 in LTD induction. The anti-apoptotic proteins XIAP and Bcl-xl inhibit LTD induction by limiting the activation of the apoptotic cascade.

system? Early findings that AMPA receptors are cleaved by caspases during excitotoxic neuronal death suggested this possibility[19]. Notably, an interesting study showed that caspase-3 is involved in AMPA receptor internalization during NMDA receptor-dependent long-term depression (LTD) in hippocampal neurons, and a serine/threonine kinase Akt1 appears to be the substrate of caspase-3 for its action in LTD through controlling glycogen synthase kinase-3β (GSK3β) activity[3] (Fig. 1B). In line with the hypothesis that the mitochondrial pathway is involved in LTD induction, over-expression of the anti-apoptotic protein XIAP or Bcl-xL[3] or down-regulation of the pro-apoptotic protein BAD or BAX inhibits LTD in CA1 neurons[20]. Differential activation of the BAD-BAX-caspase-3 cascade in LTD and apoptosis indicates that fine-tuning of caspase-3 activity during LTD induction may ensure that neurons escape from apoptosis[20]. Amyloid-β_{1-42} (Aβ) is believed to be a critical factor in causing cognitive decline in Alzheimer's disease, presumably by affecting hippocampal long-term potentiation (LTP). Recently, it has been shown that the caspase-3, AKT, and GSK3β pathway is involved in the effects of Aβ on LTP[21]. The failure in LTP-induction manifests in some ways in synapse loss, which occurs normally during development or pathologically during neurodegenerative diseases. Indeed, local activation of caspase-3 by photostimulation of mitochondria-targeted KillerRed, which triggers mitochondrial damage and activates the intrinsic pathway of apoptosis, induces local spine elimination and dendrite retraction in cultured hippocampal neurons, without inducing full apoptosis[22]. In contrast, caspase-3-knockout mice exhibit increased spine density and altered miniature excitatory post-synaptic currents. Taken together, these findings suggest that caspase-3 is involved in the elimination of postsynaptic structures in the CNS and peripheral synapses.

Is it possible that caspases also regulate presynaptic structures or functions? Indeed, we found that genetic ablation of *caspase-3* markedly restores the presynaptic structures of motor nerve terminals in agrin-knockout mice[8]. This phenomenon could be explained by the presence of either retrograde signals expressed in muscle cells or a direct role of caspase-3 in pre-synaptic differentiation. Indeed, some axon guidance factors, e.g., netrin-1 or lysophosphatidic acid, activate caspase-3 in retinal axonal growth cones and caspase-3 activity is essential for the induced chemotropic responses[7]. In addition, local caspase activity has been observed in the branch points of the axonal arbors of young retinal ganglion cells in zebrafish embryos and this pattern correlates with axon-repulsive Slit-Robo signaling[23]. Down-regulation of caspase-3 or caspase-9 increases the stability of arbors and presynaptic sites[23]. Presynaptic differentiation involves several consecutive steps, including biogenesis of synaptic vesicles, transport along axonal microtubules or actin filaments, docking to and fusion with presynaptic axonal membrane, and exocytosis and recycling of synaptic vesicles, and most, if not all, of these steps require the coordination of cytoskeletal structures. The role of caspases in the dynamics of axonal growth cones implies a potential non-apoptotic role of caspases in presynaptic differentiation and remodeling. During maturation of the NMJ, motor nerves shift from multiple innervation to single innervation, while the shape of postsynaptic structures change from plaque to pretzel-like[9]. It would be of interests to determine whether caspases participate in the terminal dynamics of motor nerves as well as the maturation of postsynaptic structures.

In summary, accumulating lines of evidence from various systems have suggested non-apoptotic roles of caspases, in particular caspase-3, in synapse refinement under physiological and pathological conditions. Identification of the mediators responsible for this tightly-controlled local apoptotic pathway is not only helpful for understanding the mechanisms of brain wiring, but is also relevant to understanding brain disorders.

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