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ORIGINAL PAPER

New Insights of a Neuronal Peptidase DINE/ECEL1: Nerve Development, Nerve Regeneration and Neurogenic Pathogenesis

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Abstract

Our understanding of the physiological relevance of unique Damage-induced neuronal endopeptidase (DINE) [also termed Endothelin-converting enzyme-like 1 (ECEL1)] has recently expanded. DINE/ECEL1 is a type II membrane-bound metalloprotease, belonging to a family including the neprilysin (NEP) and endothelin-converting enzyme (ECE). The family members degrade and/or process peptides such as amyloid β and big-endothelins, which are closely associated with pathological conditions. Similar to NEP and ECE, DINE has been expected to play an important role in injured neurons as well as in developing neurons, because of its remarkable transcriptional response to neuronal insults and predominant neuronal expression from the embryonic stage. However, the physiological significance of DINE has long remained elusive. In the last decade, a series of genetically manipulated mice have driven research progress to elucidate the physiological aspects of DINE. The mice ablating *Dine* fail to arborize the embryonic motor axons in some subsets of muscles, including the respiratory muscles, and die immediately after birth. The abnormal phenotype of motor axons is also caused by one amino acid exchanges of DINE/ECEL1, which are responsible for distal arthrogryposis type 5 in a group of human congenital movement disorders. Furthermore, the mature *Dine*-deficient mice in which the lethality is rescued by genetic manipulation have shown the involvement of DINE in central nervous system regeneration. Here we describe recent research advances that DINE-mediated proteolytic processes are critical for nerve development, regeneration and pathogenesis, and discuss the future potential for DINE as a therapeutic target for axonal degeneration/disorder.

Keywords Neuropeptide · Motor neuron · Nerve injury · Neuromuscular junction · Distal arthrogryposis · Axon degeneration

Introduction

Damage-induced neuronal endopeptidase (DINE) was originally identified as a novel nerve regeneration-associated gene (RAG), which encodes a type II membrane-bound metalloprotease belonging to M13 family [1] (Fig. 1). M13 family members include neprilysin (NEP) and endothelin converting enzyme (ECE), which are well-characterized as amyloid beta degrading enzyme and endothelin-precursor processing enzyme, respectively [2, 3]. Both enzymes are closely associated with pathological conditions such as Alzheimer's disease and the paediatric aganglionic disease known as Hirschsprung disease [4–6]. Due to structural similarity with NEP and ECE, DINE has been expected to have an important role in injured neurons, because *Dine* has a unique feature in that it is extremely up-regulated in response to both peripheral nervous system (PNS) and central nervous system (CNS) injuries (Fig. 2). Apart from our group, other research group identified XCE, a human homologue of DINE, (later termed endothelin converting enzyme-like 1 (ECEL1)) using homology cloning with ECE [7]. Despite the highly conserved amino acid sequences with NEP and ECE, DINE/ECEL1 is not likely to share the same substrates, and is still an orphan protease. Several trials to

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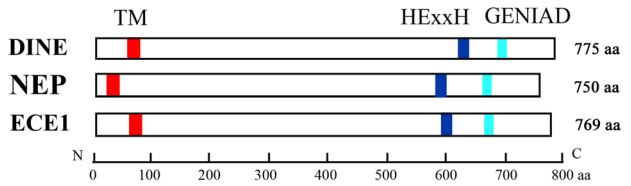

Fig. 1 Comparison of the primary structure of M13 family members. The red box and the dark blue box represent the transmembrane domain and the HExxH zinc binding domain respectively. The light blue box indicates the catalytically important GENIAD sequence

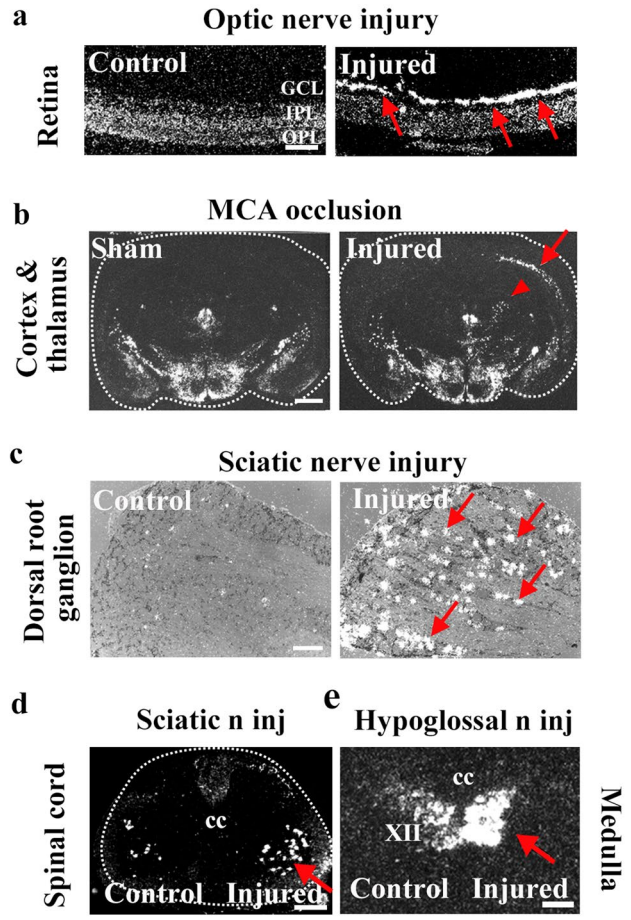

Fig. 2 The up-regulation of *Dine* mRNA after CNS and PNS nerve injuries. Arrows indicate the increased expression of *Dine* mRNA after injury. MCA, middle cerebral artery; Sciatic n inj, sciatic nerve injury; Hypoglossal n inj, hypoglossal nerve injury. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; cc, central canal; XII, hypoglossal nucleus. Scale bars, 80 μm in **a**, 1 mm in **b**, 40 μm in **c**, 170 μm in **d** and 90 μm in **e**, Figures are modified from Kiryu-Seo et al. [1]

identify its endogenous ligands were failed and the function of DINE has been obscure for a long time.

For the last decade, new genetically manipulated mice have provided an insight into DINE/ECEL1 during development, regeneration and pathogenesis in the nervous system. During embryonic development, DINE/ECEL1 contributes to axon arborization of spinal motor nerves and the subsequent formation of the neuromuscular junction (NMJ) [8, 9]. Recent human genetics have revealed that mutations of the *DINE/ECEL1* gene lead to autosomal recessive type 5 distal arthrogryposis DA (DA5), which is a group of congenital movement disorders [10, 11]. The deficiency or mutations of the *Dine* gene, which are responsible for DA5, result in the abnormal axonal branching of motor nerve and NMJ formation, demonstrating that the abnormality of motor nerves is a primary cause of DA5 [12, 13]. In addition to the significance of DINE in developing and pathogenic motor neurons, it is likely that injury-induced DINE enhances CNS nerve regeneration, as the molecular name indicates [14]. All these studies have established that DINE/ECEL1 is critical for development, regeneration/degeneration and pathological conditions.

In this review, we begin with an outline of DINE/ECEL1 and then discuss the latest advances in our knowledge obtained from genetically manipulated mice. We use the molecular name DINE in this review, instead of the nomenclature, ECEL1, from the above-mentioned our history. Protease studies are supposed to start from biochemical analysis in general. The understanding of DINE has been unexpectedly advanced by pathophysiological outputs of gene manipulated mice in the past decade. Based on in vivo studies, here we introduce the recent progress underlying the pathophysiology of DINE in vivo and discuss the significance of DINE in developing and pathogenic motor neurons as well as injured neurons.

Identification of DINE

DINE was identified as a novel RAG [1]. Over 20 years ago, there were only a limited number of RAGs. As gene profiling was thought to be a good tool to understand molecular mechanisms underlying nerve regeneration, we have searched RAGs by using differential display PCR comparing mRNA expression levels between control and injured cranial hypoglossal motor nuclei of rat, which was a unique method before powerful large-scale transcriptomic analysis methods such as microarray and RNA-sequencing were developed [15, 16]. Among the identified RAGs, there was a novel gene that showed remarkable nerve injury-responsiveness. Various kinds of nerve injuries such as traumatic brain injury, optic nerve injury, middle cerebral artery occlusion, cranial nerve injuries and sensory nerve injury induced the expression of the gene even though it was not originally expressed in the neurons (Fig. 2). This novel gene encoded a membrane-spanning type II metalloprotease carrying a typical zinc binding motif of amino acids, HELTH, and the

following conserved amino acid sequence, GENIAD, and had high homology with NEP and ECE (Fig. 1). From these unique features, we named the molecule Damage-induced neuronal endopeptidase (DINE) [1]. Apart from our group, other group found *XCE* (later registered as *ECEL1* following the nomenclature rule about "X"), which is a human homologue of *Dine*, on the basis of homology cloning using a human cDNA library with *ECE* [7], and assigned it to chromosome 2 (2q36-37) [17]. The homology between DINE, NEP and ECE predicts the similar substrates of DINE. However, ordinary biochemical assays using overexpressing cell lines failed to show protease activity of DINE against known physiological peptides such as amyloid β, big-endothelins, galanin, calcitonin and bradykinin [7, 18]. In addition, the experiments overexpressing or ablating *Dine* in cell lines and primary neuronal cultures have provided few phenotypes. As a result, the physiological role of DINE has remained elusive for a long time.

The Significance of DINE During Motor Nerve Development

Neurons in the spinal cord, hypothalamus, cranial nuclei and superior cervical ganglion show abundant DINE expression from the early embryonic stage [17, 19]. This suggests that DINE plays an important role in neuronal development and metabolism as well as in injury-induced responses. We and other group both generated *Dine*-deficient (KO) mice independently [9, 20]. The KO mice generated by both groups showed the same phenotype that they died immediately after birth due to respiratory failure. During the embryonic stage, both wild-type (WT) and KO mice are present following a Mendelian rule. However, all KO pups exhibit cyanosis and die within 30 min after birth. The phenotype of KO mice has provided us with the first evidence about the physiological significance of DINE *in vivo*. Given that DINE is specifically expressed by neurons, a deficiency of *Dine* would affect neuronal structure and/or metabolism related to the respiratory system.

The phrenic nerve is a motor nerve that innervates the diaphragm muscle and controls respiration after birth. Morphologically, the neuronal cell body of the phrenic nerve is located at the cervical spinal cord and extends the nerve to the diaphragm. After the phrenic nerve reaches the branching point at the diaphragm, it begins to repeat arborization to make a fun shape and eventually forms massive number of NMJ where acetylcholine receptors (AChR) are abundantly localized [21]. Considering that DINE is abundantly expressed in developing spinal motor neurons including phrenic motor neurons, it might be reasonable that *Dine* KO embryos fail to arborize the phrenic motor nerve after reaching the diaphragm and to form an appropriate NMJ (Fig. 3). The incomplete innervation of *Dine*-deficient motor nerves has also been found in intercostal muscles and a back muscle termed the latissimus dorsi [9]. In this regard, DINE would be critical for motor nerve development. The abnormality of the phrenic motor nerve was concluded as the primary cause for the deaths of DINE KO mice after birth.

Curiously, the overexpression and deficiency of DINE in culture systems such as motor neuron culture did not reproduce the abnormality of neurite behaviour. Again, DINE studies suffered from the difficulty to examine the functional significance of DINE in vitro culture systems. Why are in vitro culture systems unable to reproduce in vivo phenotypes? One possibility is the lack of substrates in vitro culture systems. The other possibility is that DINE requires a co-factor or specific functional subcellular domain to be fully activated.

Does DINE Function as a Protease?

Studies using *Dine* KO mice clarified that DINE was essential during development in vivo. Next question is how DINE functions physiologically. As DINE belongs to the M13 family that includes NEP and ECE, which degrade/process peptides such as amyloid β, enkephalin, big-endothelins and galanin, DINE was predicted to cleave similar neuropeptides from the homology between family proteins. However, our and other group's trials using artificial and physiological peptides failed to detect the proteolytic activity of DINE [1, 17, 18]. The data raised a question as to whether DINE functions as an adhesion molecule rather than a protease. To address the issue, an in vivo rescue experiment was designed because neither the protease activity nor the apparent phenotype were detected in an in vitro culture system. The experiment initially generated a couple of transgenic mice expressing wild-type DINE or the two types of mutant DINE which encode a deleted HELTH motif and a mutated Glu672 or a single point mutated Glu613. These exogenous proteins are expressed specifically in embryonic motor neurons under the control of the embryonic motor neuron-specific *Hb9* promoter. The transgenic mice are then crossed with *Dine* KO mice [8] (Fig. 3). The *Dine* KO mice carrying the wild-type *Dine* transgene rescued the abnormality of phrenic nerve arborization and NMJ formation, while those carrying mutant *Dine* transgenes failed to do this (Fig. 3). These in vivo rescue experiments clearly showed that proteolytic activity of DINE is critical for motor nerve arborization during the embryonic stage.

The other hallmark is that DINE influences cell–cell communication through protease activity, in this case, the axon and immature Schwann cells. The developing phrenic motor nerve extends the axon to the diaphragm while Schwann cell precursors (SCPs) independently

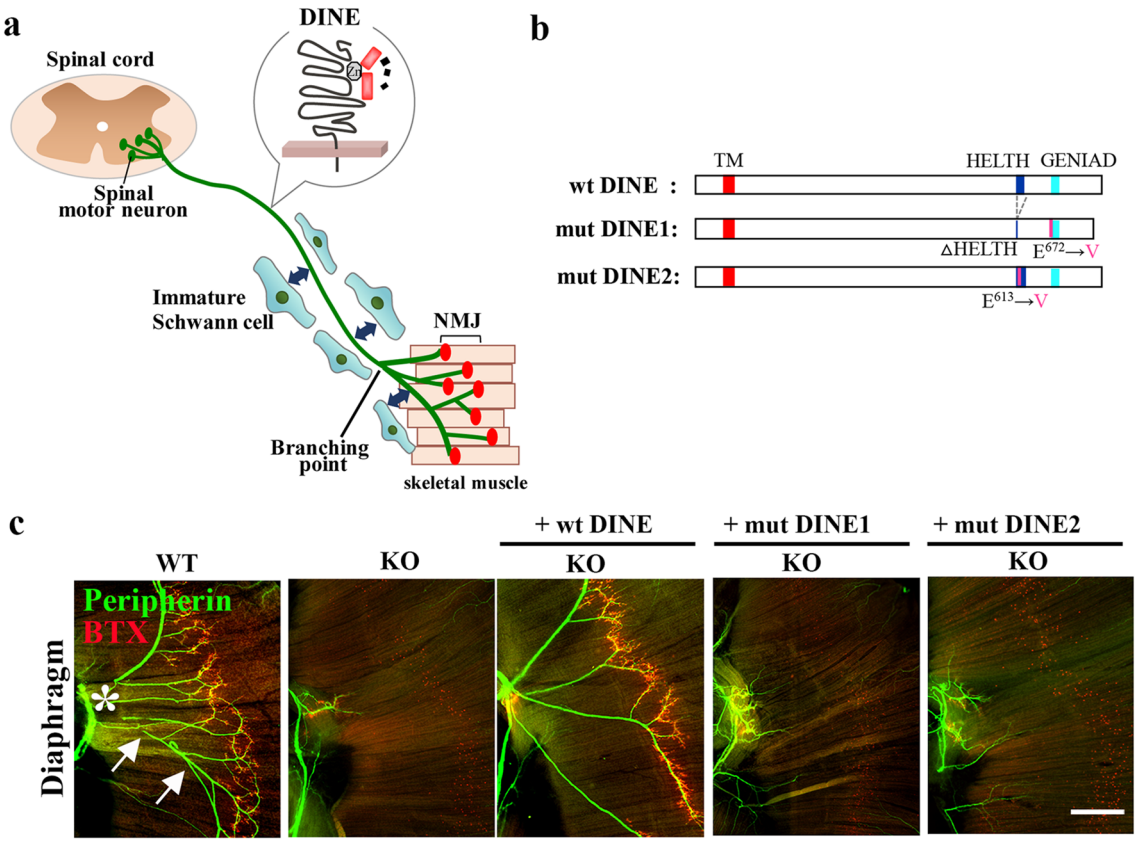

Fig. 3 Axon arborization of motor nerves during embryonic development. **a** Schematic illustration of motor nerve extension and arborization during the embryonic stage. **b** Types of exogenous DINE specifically expressing in developing motor neurons in our study c. TM; transmembrane domain, HELTH; zinc-binding motif, GENIAD; conserved consensus sequence. **c** Arborization of phrenic motor nerve at embryonic day 17. Phrenic motor nerve and NMJ were stained with peripherin (green) and α-bungarotoxin (BTX, red) respectively. The exogenous protein of wt DINE, mut DINE1 or mut DINE2 shown in b is expressed in embryonic motor neurons of the *Dine* KO mouse. Arrows show phrenic motor nerves. Asterisk denotes the branching point of the phrenic nerve. Scale bar, 500 μm. Images are modified from Nagata et al. [9] and Matsumoto et al. [8]

co-migrate along the axon [22, 23]. After innervating the diaphragm, the axon interacts with SCPs, which differentiate to immature Schwann cells and align along the axon to initiate myelination [24, 25]. SCPs and immature Schwann cells then begin to influence axonal development of the phrenic motor nerve. ERBB2/3 and SOX10 reside in Schwann cells and function as receptors for axonal signals and as a transcription factor, respectively. Both play key roles in Schwan cell development. Mice lacking SCPs, such as *Erbb*2/3 or *Sox10*-deficient mice, exhibit similar but more severe phenotypes of phrenic nerve compared with *Dine* KO mice [26–28]. In this context, axonal DINE seems to influence the behavior or differentiation of neighboring immature Schwann cells, presumably through the cleaved peptides, to promote axonal branching and NMJ formation.

Transcriptional Response of DINE to Nerve Injuries

The most intriguing property of DINE is an extreme transcriptional response against kinds of nerve injuries [1] (Fig. 2). The induction of *Dine* expression is seen in response to motor and sensory nerve injuries, brain and spinal cord trauma, and cerebral ischemia. Furthermore, *Dine* is often ranked among the most up-regulated genes in transcriptome analyses using various injury models [29–33]. The enhanced *Dine* expression is restricted to neuronal cells and is not seen in glial cells. It is likely that mechanical and chemical injury of neuronal axons may be required to induce *Dine* mRNA expression. In the ischemic brain, *Dine* mRNA is expressed in a group of

neurons projecting their axons to and/or through the core region of the infarction, which suggests that DINE protects the secondary degenerative death of neurons in the peri-infarct area [34].

The expression change of *Dine* mRNA in the dorsal root ganglion (DRG) provides further insights about the role of DINE. In non-injured DRG, few *Dine* mRNA-expressing neurons were observed; however, after sciatic nerve injury, the expression of *Dine* mRNA markedly increased, mainly in small-sized DRG neurons [35]. More than 80% of *Dine* mRNA-positive neurons simultaneously expressed the neuropeptide, Galanin. Interestingly, the application of leukemia inhibitory factor (LIF) and the nerve growth factor (NGF)-neutralizing antibody to intact sciatic nerves induced the mRNA expression of both *Dine* and *Galanin* in DRG neurons, which suggests that the expression of both *Dine* and *Glanin* is similarly regulated at the downstream of LIF-gp130 and JNK signaling pathways after nerve injury [35]. These findings suggest that Galanin could be a potential candidate as a substrate for DINE. However, as mentioned above, this was not the case. Apart from the LIF-gp130 and JNK signaling pathways, the dual leucine zipper kinase (DLK) is also located at the upstream of *Dine* transcriptional regulation in injured DRG. DLK is a stress-sensitive molecule and is associated with axonal regeneration/degeneration [36–38]. A recent report by Waschin et al. showed that *Dlk*-deleted mice were protected from allodynia following sciatic nerve injury and that *Dlk*-ablated DRG neurons decreased the expression of *Dine* mRNA after nerve injury [39], which suggests that DINE is involved in the establishment of neuropathic pain after nerve injury. The change of *Dine* mRNA in DRG is also observed in other models. Fabry disease is an X-linked lysosomal storage disorder. The accumulation of glycosphingolipids within peripheral nerves and DRG results in pain derived from small-fiber neuropathy. The α-galactosidase A deficient mouse is known as Fabry disease model mouse. The α-galactosidase A deficient DRG neurons have enhanced the expression of *Dine* mRNA, compared with those of wild type mouse [40]. All these findings propose the possibility that DINE is up-regulated in damaged DRG and could be associated with neuropathic pain.

The transcriptional mechanism of *Dine* in response to nerve injury is an important issue to examine how DINE is involved in nerve regeneration. We found that unique transcriptional machinery exists in response to nerve injury, in which the general transcription factor, Sp1, binds to the GC-box in the *Dine* promoter and functions as a scaffold protein to recruit cJun, ATF3 and STAT3, which have been accepted as nerve regeneration associated transcription factors [41]. This mechanism, which does not require so many specific binding sites for transcription factors would be practical and effective in the up-regulation of many genes after nerve injury. Recent large-scale bioinformatic analyses have identified several hub-transcription factors that activate nerve regeneration programmes [42]. These hub transcription factors include ATF3, cJun, Stat3 and Sp1, that form the transcriptional complex in the *Dine* promoter in response to nerve injury. Of the complex, activating transcription factor 3 (ATF3) seems to be the most critical in nerve injury, because ATF3 is specifically expressed after nerve injury [43, 44]. Indeed, the expression of DINE is well co-localized with that of ATF3 in various nerve injury models [34, 35, 41, 45]. These studies emphasize that DINE locates at the pivotal position for the initiation of the nerve regeneration programme. Collectively, it is valuable to explore the physiological role of DINE in injured neurons.

The Impact of DINE on Axon Regeneration/Degeneration

Mammalian CNS neurons have limited ability to regenerate [46, 47]. Like other CNS neurons, the retinal ganglion cells (RGCs) hardly regenerate after optic nerve injury. Healthy RGCs never express moderate level of DINE under a normal state, while injured RGCs show enormous up-regulation of DINE expression (Fig. 4). The expression time course of DINE is coordinated with that of ATF3. Injured RGCs down-regulate the expression of these molecules at an earlier phase, probably because of a decline in regenerative ability [14]. Many researchers have looked for molecules or reagents that can promote nerve regeneration after optic nerve injury and have partially succeeded in extending axons beyond the injury site. Among them, zymosan, which is a potent monocyte activator, is a well-known regeneration-promoting reagent [48]. Zymosan treatment has actually enhanced and prolonged the expression of DINE and ATF3 in injured RGCs, suggesting that the regenerative potency is activated.

DINE has been expected to be involved in nerve regeneration events owing to its unique transcriptional response to injury. However, the lethality of the *Dine* KO mouse has not allowed us to examine the functional significance of DINE in adult injured neurons for a long time. Recently, we have succeeded in obtaining adult *Dine* KO mice. The *Dine* KO mice express exogenous wild-type DINE specifically in embryonic motor neurons, which we describe in the in vivo rescue study in the previous section, and not only rescued the abnormal arborization of the motor nerve but also prevented the lethality of *Dine* KO mice [8]. The rescued *Dine* KO mice survive after birth and develop normally even though the expression of rescue transgenes has been abolished after postnatal day 8. The adult KO mice show normal NMJ formation and motor performance (our unpublished data), meaning that the deficiency of *Dine* does not affect the maintenance and homeostasis of motor neurons and axons. However, the adult *Dine* KO mice have

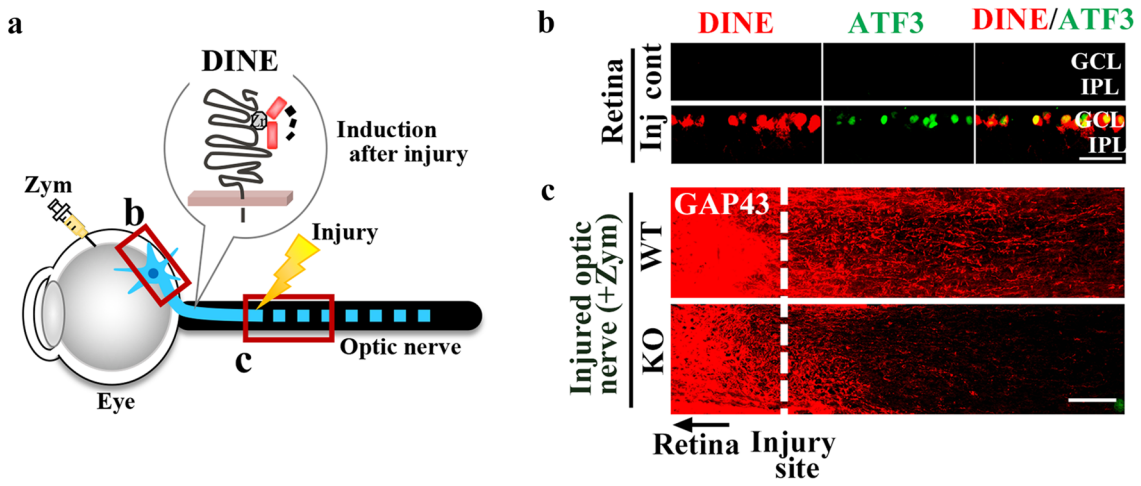

Fig. 4 The significance of DINE in optic nerve regeneration. **a** Experimental optic nerve regeneration. Injured retina and optic nerve are shown in **b** and **c** respectively (red box). **b** Immunostaining of DINE and ATF3 in control and injured retina. c. Injured optic nerve of the wild type mouse (WT) can regenerate in the presence of zymosan (zym), while that of KO cannot. GAP43 immunoreactivity shows regenerating axons. GCL, ganglion cell layer; IPL, inner plexiform layer. Scale bars, 50 μm in b and 100 μm in c. Images are modified from Kaneko et al. [14]

shown a failure to extend the axon after optic nerve injury even in the presence of the regeneration-promoting reagent zymosan [14] (Fig. 4). It is probable that the peptide proteolyzed by DINE impacts the ability of the injured axon and/or surrounding glial cells to promote nerve regeneration. The study by Kaneko et al. [14] has answered an old and simple question as to whether injury-induced DINE functions in damaged neurons. Recently, Sato et al. have reported that knockdown of DINE in injured retinal ganglion cells (RGCs) using AAV-mediated CRISPR/Cas9 system accelerates RGC death after optic nerve injury [49]. Although the exact mechanism of DINE in damaged RGCs require further study, these studies confirmed that DINE is actually involved in nerve regeneration.

Exploring the DINE-mediated pathway including substrates would provide insight into a novel therapeutic target for a broad range of neuronal damages including traumatic injuries, neurodegenerative diseases, neuropathic pain, diabetic neuropathy and tissue damage.

Mutations in Human DINE/ECEL1 are Associated with a Type of Distal Arthrogryposis

To examine further significance of DINE, it is also important to understand the role of DINE in the context of human pathology. Accumulated evidence demonstrates that metalloproteases of the NEP family have a number of physiological substrates in various human cells and tissues [50]. Disruption and/or altered enzymatic activity of the proteases are therefore linked with some diseases possibly via the abnormal processing of its physiological substrates. For example, a loss-of-function mutation in the *ECE* gene has been associated with Hirschsprung disease, a developmental intestinal disorder characterized by the absence of ganglia in the distal colon [51]. More recently, whole-exome sequencing analysis has identified *NEP* as a genetic cause of autosomal recessive Charcot-Marie-Tooth (CMT) disease type 2, one of the most common forms of inherited peripheral neuropathies [52]. One variant in *NEP* has also been linked with an autosomal dominant spinocerebellar ataxia [53].

As for DINE, it has remained unclear for over 10 years whether DINE plays a role in certain pathological conditions in humans. At the end of 2012, two different groups reported that mutations in the *ECEL1* gene (human orthologue of *Dine*) caused a congenital movement disorder termed DA5 [MIM 615065] [10, 11]. One group led by Joel Lunardi performed genomic mapping and single nucleotide polymorphism (SNP) genotyping in a genome-wide manner to search for the possible DA locus in consanguineous families. Subsequent Sanger sequencing enabled them to narrow down their candidate genes, and finally identify seven genetic variants in the *ECEL1* gene as causal DA mutations [10]. At the same time, another group identified nine pathogenic mutations using linkage analysis and whole genome sequencing [11]. Like other DA patients, *ECEL1*-mutated DA patients commonly display multiple joint limitations especially in the distal parts of their limbs from birth onward. These two initial studies showing the pathogenic consequences of *ECEL1* mutations were successfully reproduced in subsequent studies. Up to the present, 26 mutations in total have been identified as pathogenic mutations [10, 11, 54–63]. They contain four nonsense mutations and three frameshift mutations,

both of which lead to loss of full-length ECEL1 proteins, indicating that loss-of-function of ECEL1 could lead to the onset of DA. Moreover, 11 missense mutations, which lead to only one amino acid exchange, have been identified to date. These missense mutations of the *ECEL1* gene in DA are summarized in Fig. 5. Among these missense mutations, three mutations seem to be associated with the abnormal enzymatic activity of DINE/ECEL1 from the position of mutations. A missense mutation C760R (replacement of Cys760 with Arg) is expected to affect the catalytic process of DINE through the malformation of a disulfide bond with a cysteine residue [10]. A675T and G607S are other missense mutations positioned adjacent to the zinc binding motif essential for enzymatic activity [56, 59]. In silico conformational analyses based on the extracellular protein structure of a family member provided the possibility that the pathogenic missense mutation blocks the Zn binding in the putative active site of DINE/ECEL1 [56].

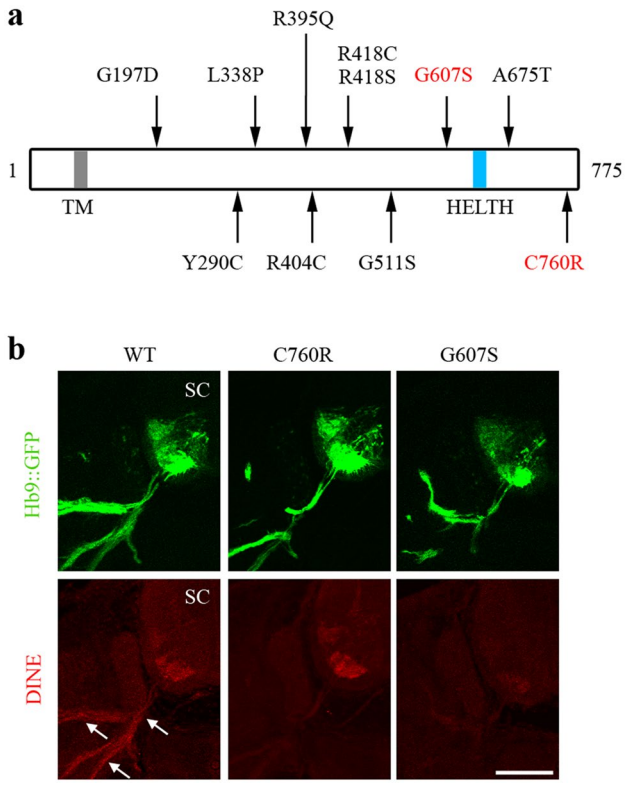

Fig. 5 *DINE* missense mutations as a cause of distal arthrogryposis. **a** All reported pathogenic missense mutations are presented in the DINE/ECEL1 protein structure (775 amino acid). The corresponding mutations that we introduced into our mouse models are shown in red. **b** Immunohistochemical analysis with anti-DINE antibody in horizontal sections of E12.5 mouse spinal cords from wild-type, homozygous C760R mutant and homozygous G607S mutant embryos. Arrows indicate DINE-positive motor nerves. Scale bar, 200 µm

DINE Mutant Mice as Relevant DA Models

Human clinical studies have provided convincing evidence that pathogenic mutations in the *ECEL1* gene are causes of DA, but it remains unclear how the pathogenic mutations lead to the congenital disorder with limited movement in the limbs. It is infeasible to obtain biopsy samples from patients with movement disorders who are otherwise healthy to further explore the etiology of *ECEL1*-mutated DA. Given that the amino acid sequence of DINE/ECEL1 is highly conserved with 95% identity between mice and humans, *Dine* KO mice seem to be a suitable model for resembling DA patients with *ECEL1* loss-of-function mutations. We crossed a *Dine* KO mouse with a reporter mouse line, *Hb9*-GFP Tg mouse, to visualize embryonic cranial and spinal motoneurons and their nerves by GFP. The various stages of *Dine*-deficient embryos have shown the aberrant axonal arborization of motor nerves in over 10 limb muscles [12]. The abnormality of the presynaptic motor nerves subsequently resulted in the prominent reduction of innervated AChR clusters. These results provided a possibility for the first time that disruption of DINE/ECEL1 function could result in the onset of DA possibly via the motor innervation defects in embryonic limb muscles.

Among DA-responsible genetic mutations of *ECEL1*, we further focus on two types of missense mutations, C760R and G607S, which are implicated in the loss of enzymatic activity as described in the previous section. We attempted to explore whether these *ECEL1* missense mutations affected motor nerve development in limb muscles. To this end, we directly introduced each DA missense mutation in the endogenous mouse *Dine* locus into mouse zygotes using genome editing technology termed CRISPR/Cas9, and then performed detailed morphological analyses. The homozygous C760R mutant mice showed the same axonal arborization defects of limb motor nerves as *Dine* KO mice, supporting the suggestion that motor innervation defects could be a primary cause of *ECEL1* mutated DA [12]. Interestingly, subsequent immunohistochemical analyses with anti-DINE antibody revealed the altered expression pattern of the DINE protein in embryonic spinal motoneurons (Fig. 5b) [13]. Unlike the wild type protein, the C760R mutant protein was localized only in the cell soma but not in the axons. These data were reproduced in another mutant mouse line with an artificial mutation C760G, demonstrating that the position (i.e. Cys760) of the mutation is more important than the specific amino acid substitution. Contrary to our *in vivo* data, C760R mutant proteins did not alter the intracellular localization in cultured COS-7 cells [10]. This discrepancy is likely due to the different experimental paradigms; between in vivo spinal motor neurons and in vitro non-neuronal cultured cells, or between the targeting mutation into the endogenous *Dine* locus and the overexpression

of the mutant protein. The subcellular localization of DINE still remains a mystery, although previous report has shown that DINE resides mainly the endoplasmic reticulum using cell lines [64]. The result of C760R mutant mouse proposes that the proper subcellular localization of DINE is critical for its function. How is DINE localized in axons, for example by the transport of the endosome containing DINE to the axonal terminal or the translocation of DINE onto the cell surface? Further study will be required to answer the question. Additional *Dine* mutant mice carrying a G607S missense mutation showed a drastic decrease of *Dine* mRNA in embryonic spinal motor neurons. This is likely to be caused by the abnormal splicing process but not the enzymatic loss (Fig. 5b) [13]. These results emphasize that genome editing based generation of *Dine* knock-in mice with pathogenic mutations is quite useful to capture the functional annotation of pathogenic mutations. Further in vivo exploration for pathogenic *ECEL1* mutations (especially A675T) would be necessary for understanding the function of DINE/ECEL1 as a peptidase.

Future Directions

Recent studies using genetically manipulated mice have driven our understanding about the functional significance of DINE. Axonal extension during development and regeneration often shares a similar mechanism. In line with this, the abnormal arborization of developing motor axons in *Dine* KO or pathogenic mutant mice and the regeneration failure of injured axons in adult *Dine* KO mice may be due to the lack of a similar mechanism with each other in which the axonal DINE influences or communicates with surrounding glial cells probably via cleaved peptides. There is still a missing link between the molecular existence of DINE and its physiological outputs. Extensive future work is required to identify substrates, the molecular interaction with other proteins and the DINE-mediated signalling pathway. The subcellular localization of DINE is an additional important issue. Although DINE is considered to mainly localize in the endoplasmic reticulum as shown in a previous study using cell lines [64], we cannot rule out the possibility that DINE requires the subcellular alteration to be active, for example the transport of the endosome containing DINE to the axonal terminal or the translocation of DINE onto the cell surface. The mislocalization of pathogenic C760R mutant DINE proteins may support this possibility. The challenges to address these questions would provide physiological and pathological understanding of DINE and a new insight for regenerative medicine, pathogenic clinical therapy and drug design.

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Conflict of interest The authors declare that they have no conflict of interest.

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