Neuronal necrosis and spreading death in a Drosophila genetic model

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Brain ischemia often results in neuronal necrosis, which may spread death to neighboring cells. However, the molecular events of neuronal necrosis and the mechanisms of this spreading death are poorly understood due to the limited genetic tools available for deciphering complicated responses in mammalian brains. Here, we engineered a Drosophila model of necrosis in a sub-population of neurons by expressing a leaky cation channel in the Drosophila eye. Expression of this channel caused necrosis in defined neurons as well as extensive spreading of cell death. Jun N-terminal kinase (JNK)-mediated, caspase-independent apoptosis was the primary mechanism of cell death in neurons, while caspase-dependent apoptosis was primarily involved in non-neuronal cell death. Furthermore, the JNK activation in surrounding neurons was triggered by reactive oxygen species (ROS) and Eiger (Drosophila tumor necrosis factor x (TNFα)) released from necrotic neurons. Because the Eiger/ROS/JNK signaling was also required for cell death induced by hypoxia and oxidative stress, our fly model of spreading death may be similar to brain ischemia in mammals. We performed large-scale genetic screens to search for novel genes functioning in necrosis and/or spreading death, from which we identified several classes of genes. Among them, Rho-associated kinase (ROCK) had been reported as a promising drug target for stroke treatment with undefined mechanisms. Our data indicate that ROCK and the related trafficking pathway genes regulate neuronal necrosis. We propose the suppression of the function of the trafficking system, ROS and cytokines, such as TNFα, as translational applications targeting necrosis and spreading death.

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As the second leading cause of death worldwide, brain ischemia often results in necrotic cell death in the infarct core and cellular dysfunction in the peri-infarct zone known as ischemic penumbra. Because dysfunctional cells in the penumbra may be rescued through prompt reperfusion within a few hours after the insult, penumbral salvage has been the major aim of therapy. However, most patients are admitted to clinics many hours after the insult, resulting in permanent damage in the penumbra. In such cases, alleviation of cell death and inflammation may be more critical for recovery. Indeed, some patients suffered from progressive dementia even years after the insult. Currently, no effective neuroprotective therapy has been developed. One reason is that our limited knowledge of cell death restricts the design of treatment strategies.

In ischemia and reperfusion, extremely complicated cellular and molecular events induce acute and progressive cell death, including excitotoxicity, energy loss, Jun N-terminal kinase (JNK) activation, generation of reactive oxygen species (ROS), release of apoptosis-inducing factor (AIF) and cytokines (such as tumor necrosis factor α (TNFα)), metabolic changes, protein aggregation, organelle damage and synaptic failure. Sometimes, the causal or responsive roles of these changes on cell death are hard to dissect. Many questions remain, such as how adjacent cells respond to the necrotic infarct core, what death signals are released to spread death, and whether there are genetic pathways regulating cell death.

Here, we built a genetic system to induce necrosis in a sub-population of neurons in Drosophila, and we studied its effect on neighboring cells. To induce neuronal necrosis, a leaky cation channel was expressed to overload the cells with calcium. Our data indicate that neuronal necrosis results in extensive spreading of death to adjacent cells through complicated signaling pathways, including ROS, JNK, Eiger (Drosophila TNFα) and other unknown signals. Furthermore, these spreading death signals are also required for cell death induced by hypoxia and oxidative stress, suggesting that they are relevant to ischemic stroke in mammals. Moreover, we performed large-scale genetic screens and identified several classes of proteins that may regulate primary necrosis and/or spreading death. Our model provides a genetic tool to dissect the molecular mechanisms of necrosis and spreading death, which may mimic the molecular events in the ischemic brain.

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Abbreviations: GluR1Lc, glutamate receptor 1 Lurcher mutant; sev, sevenless; TNFα, tumor necrosis factor α; ROS, reactive oxygen species; JNK, Jun N-terminal kinase; PI, preserved iodide; AO, acridine orange; DHE, dihydroethidium; AIF, apoptosis-inducing factor; IAP, inhibitor of apoptosis protein; JNKK, JNK Kinase; JNKKK, JNKK Kinase; TEM, transmission electron microscopy; ROCK, Rho-associated kinase; GOF, gain-of-function; LOF, loss-of-function; a.p.f., after pupa formation

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Results

Expressing a leaky cation channel in a subset of neurons in the *Drosophila* eye caused extensive cell death and eye defects. To induce neuronal necrosis, we generated a transgenic fly line to express a constitutively open cation channel, the mouse glutamate receptor 1 *Lurcher* mutant (*GluR1<sup>Lc</sup>). Then, *UAS-GluR1<sup>Lc</sup>* was expressed in fly eyes, driven by the sevenless-Gal4 (*sev-Gal4*) promoter. The fly progenies from *sev-Gal4* and *UAS-GluR1<sup>Lc</sup>* are simplified as *sev-GluR1<sup>Lc</sup>* (the binary UAS/Gal4 expression system is represented as ‘>’ throughout the text). The compound eyes of *Drosophila* are formed by nearly 800 units of small eyes, known as ommatidia, each of which contains 8 photoreceptor cells (or R cells). During development in the larval eye disc, R8 recruits the R2/R5 pair and the R3/R4 pair, and they form a five-cell pre-cluster. In the adult stage, the R1/R6 pair and R7 are also recruited into the ommatidium. The *sev-Gal4* promoter is specifically expressed in the R3/R4 pair of the larval eye disc and R3/R4/R7 of the adult eye. In the *GluR1<sup>Lc</sup>*-expressing larvae, we verified that the transgenic GluR1<sup>Lc</sup> protein was located in the plasma membrane (Supplementary Figure S1A) and that calcium overloading by GluR1<sup>Lc</sup> indeed occurred in *Drosophila* neurons (Supplementary Figure S1B).

In *sev-GluR1<sup>Lc</sup>* flies, the adult eye size was greatly reduced (Figures 1Aa and b), as were the numbers of ommatidia and bristles (Figures 1Ac–d). Strikingly, few cells were identifiable in the cross-sectioned ommatidia (Figures 1Ae and f). By transmission electron microscopy (TEM), the damaged cells exhibited loss of plasma membrane integrity and emergence of intracellular vacuoles (Figures 1Ag and h). These results suggest that massive death occurred in neuronal and non-neuronal cells in the adult eyes. At the larval stage, the GFP fluorescent intensity in the eye disc of the *sev-GluR1<sup>Lc</sup>*/*eGFP* (*UAS-eGFP* could visualize the *GluR1<sup>Lc</sup>*-expressing cells) flies was greatly reduced (Figure 1B), suggesting that cell death occurred in the third instar larval stage, after the *sev-Gal4* promoter began to express.

Occurrence of neuronal necrosis in *GluR1<sup>Lc</sup>*-expressing neurons. The death of *GluR1<sup>Lc</sup>*-expressing neurons was not mediated by caspase-mediated apoptosis because caspase inhibition had no effect on the eye defect (Figure 1C). Consistently, caspase activity could not be detected by anti-cleaved-caspase immunostaining in the larval eye disc (Figure 1D). In contrast, there was positive staining for the necrotic marker propidium iodide (PI) in the *GluR1<sup>Lc</sup>*-expressing neurons (Figure 1E). In addition, we examined other features of necrosis, including mitochondrial defects, elevation of ROS level and increase in cytoplasmic acidification, all of which took place in the *GluR1<sup>Lc</sup>*-expressing neurons (Supplementary Figure S1C; Figures 1F and G). Together, these results suggest that the *GluR1<sup>Lc</sup>*-expressing neurons die from necrosis.

Spreading apoptotic death in adjacent cells induced by primary neuronal necrosis. Because the *sev-Gal4* promoter drives *GluR1<sup>Lc</sup>* expression in two of the five R cells in larvae and three of the eight R cells in adult in each ommatidium, the other R cells should stay alive. However, the remaining number of neurons was far lower than expected (Figure 1Aa), suggesting the occurrence of spreading death. One caveat is that spreading death may be mediated through gap junctions because the R cells can form gap junctions during development. We think this scenario is unlikely because only *GluR1<sup>Lc</sup>*-expressing cells undergo necrosis. In addition, we found that mutants of several gap junction proteins did not affect fly eye size ablation (Supplementary Table S1).

To determine whether the additional deaths were from neurons or glial cells, the larval eye discs were stained with a neuronal marker (22C10, which stains the cytoskeleton) and a glial marker (Repo, which stains the nucleus). We found that spreading death mainly occurs in the posterior region of eye discs, which contains mostly mature neurons (Figures 2Aa–d). To further confirm primary (*GluR1<sup>Lc</sup>*-expressing neurons) or spreading (non-*GluR1<sup>Lc</sup>*-expressing) death, we co-stained the samples with anti-GluR1 and anti-ELAV (which stains neuronal nuclei) (Figures 2Ba–c1). We observed that the newly formed ommatidia in the anterior region of *sev-GluR1<sup>Lc</sup>* eye discs were relatively normal (Figures 2Bd and ff). However, in the posterior region, the ELAV staining was diminished in the GluR1-positive R3/4 cells (Figure 2Bf), and it became clumpy in the adjacent neurons (Figures 2Bf2 and f3). These results clearly show that spreading death occurs in adjacent neurons at the larval stage.

In addition, neuronal apoptosis induced by *sev-rpr* could not spread death in the eye disc (Figures 2C and D). Therefore, only necrosis could spread death.

Caspase-dependent spreading apoptosis in non-neuronal cells. Although caspase activation was minimal in the third instar eye disc (Figure 1D), we found that inhibition of caspasas in all ommatidial cells by GMR-P35 strongly rescued the adult eye size defect (Figures 3Aa and b). Similar effects were also observed in the double mutants of effector caspasas Dcp-1 and drICE (Figure 3Ac), and in a deficiency line (*H99*) that simultaneously removes the three inhibitor of apoptosis protein (IAP) antagonists, hid, rpr and grim (Figure 3Ad). Further analysis using *hid<sup>Avr+X1</sup>, rpr<sup>87</sup>* or either one in combination with *H99* (Figures 3Ae–h) suggests that the caspase-dependent apoptosis is mediated by *hid*. To determine which cell type was rescued by GMR-P35, we sectioned adult eyes. Non-neuronal cells, but not R cells, were strongly rescued (compare the numbers of white vacuoles in Figures 3Bb and c). We quantified the R cell salvage by ‘ommatidium density,’ the total number of identifiable ommatidia in a fixed area (1000 μm²). This quantification showed that the ommatidium density in wild type, *sev-GluR1<sup>Lc</sup>* and GMR-P35; *sev-GluR1<sup>Lc</sup>* flies was 7.63, 0.81 and 1.34, respectively (Figure 3Bd). This result suggests that caspase-dependent apoptosis accounts for ~8% (1.34–0.81/7.63–0.81) of spreading death in neurons. We exclude the possibility that one copy of GMR-P35 might be too weak to inhibit caspase activity (Supplementary Figure S2A). Furthermore, we observed that cell death markers, including acridine orange (AO) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL),
could positively label many cells in the larval eye discs, and their staining patterns were unaltered by GMR-P35 (Figure 3C). This suggests that extensive apoptosis is caspase independent. We further verified that TUNEL staining mainly labels the apoptotic cells and not the necrotic cells (co-staining of TUNEL with PI or GluR1Lc-expressing neurons, Supplementary Figure S2B).

JNK-mediated caspase-independent spreading apoptosis in neurons. To study how caspase-independent apoptosis is activated, we examined two pathways that are reported to not respond to the caspase inhibitor P35 in Drosophila, the JNK and AIF pathways. 15,16 JNK signaling requires a cascade of protein kinases, including JNK, JNK Kinase (JNKK) and JNKK Kinase (JNKKK). 17 We found that hep1/Y,
hepr75\(^+/+\) (JNKK mutants), bsk\(^+/+\) (a JNK mutant) and TRAF2\(^{Ex}\) (a mutant in a component upstream of JNKKK) all rescued the eye defects of sev->GluR1\(^{Lc}\) (Figures 4Aa–f). In contrast, AIF\(^{KO}/+\) (AIF knockout) showed no effect (Figure 4Ag). In addition, the puc\(^{E69}\) allele, a JNK gain-of-function (GOF) mutant,\(^{18}\) enhanced the eye defect (Figure 4Ah). However, expressing bsk\(^{DN}\) (dominant negative) or hepr\(^{RNAi}\) (Figures 4Ai and j) in GluR1\(^{Lc}\)-expressing neurons had no effect, indicating that JNK signaling acts in adjacent cells. We further confirmed that JNK-mediated death was caspase independent by testing the effect of bsk\(^1\) in the presence of GMR-P35, which showed additional rescue compared with GMR-P35 alone (Figures 4Ak and m). Consistently, puc\(^{E69}\) could further enhance the GMR-P35:sev->GluR1\(^{Lc}\) phenotype (Figure 4Al). Quantification of the ommatidia density suggests that \(~47\%\) of spreading apoptosis in R cells can be rescued by the hepr\(^{75}\) and bsk\(^1\) double mutants (Figure 4Bd), suggesting that JNK-mediated apoptosis is the major form of spreading death in neurons. Moreover, JNK signaling was indeed activated in the larval stage (Figures 4Ca and c) and further elevated at the pupal stage,
as determined by a JNK reporter in vivo, puc-lacZ (Figures 4Cb and d). Because anti-β-Gal was partly colocalized with anti-ELAV (Figures 4Ce and e1) but not with anti-GluR1 (Figure 4Cf), we conclude that JNK activation only occurred in adjacent neurons.

**JNK-mediated apoptosis was triggered by the release of Eiger and ROS from adjacent neurons undergoing primary necrosis.** Previous studies suggest that JNK-mediated death can be activated by eiger expression driven by a pan-eye promoter GMR-Gal4 (GMR > eiger) in Drosophila. Strikingly, when driven by the sev-Gal4 promoter, UAS-eiger (sev > eiger) failed to elevate the puc-lacZ intensity or generate an eye defect (Figure 5Ae). Actually, the lacZ intensity was slightly reduced (see also later text), indicating that eiger expression was not sufficient for JNK activation. However, overexpression of eiger in necrotic neurons (sev > GluR1/+/eiger) enhanced the puc-lacZ intensity and eye size defect (Figure 5Ad). Knocking down eiger in necrotic neurons with eigerRNAi (sev > GluR1/+/eigerRNAi) significantly diminished the elevated puc-lacZ level and rescued the eye size defect (Figures 5Ae–c) without affecting the primary necrosis (Supplementary Figure S3Ac and d). Together, these results suggest that eiger from necrotic neurons serves as a spreading factor to activate JNK signal in the adjacent cells.

Stroke-induced oxidative stress is well known to cause neuronal death. We found that expressing ND75RNAi (inhibiting mitochondrial complex I to increase ROS production) in necrotic neurons enhanced ROS production (determined by dihydroethidium (DHE) staining, Figures 5Ba and b) and activated JNK signaling (Figures 5Bc and d). Functionally, overexpression of catalase (ROS chelating enzyme) or the combination of catalase with GTPx-1 (another ROS chelating enzyme) reduced the eye defect (Figures 5Ca–c) and JNK activation (Figure 5Cd) without affecting primary necrosis (Supplementary Figures S3b and d). This suggests that ROS from necrotic cells is also required for JNK-mediated apoptosis.

**Intracellular ROS is required for cell-autonomous apoptosis but not for spreading apoptosis.** A previous report suggested that intracellular ROS levels are elevated in Drosophila photoreceptor neurons. In addition, the JNK-mediated cell death might be apoptotic or necrotic depending on cell types and stimuli. Our results demonstrate that Eiger-mediated death in R cells is caused by apoptosis, based on negative PI staining (Supplementary
Figure S3B) and mutants of necroptosis components, including loss-of-function (LOF) PGMAM5 and Drp25 (Supplementary Figure S3C). Next, we examined the functional role of ROS in spreading death, and found that chelating ROS by overexpression of catalase or GTPx-I strongly suppressed Eiger-induced death (Supplementary Figures S3Da–e). Therefore, intracellular ROS is a key effector of Eiger-mediated death. In contrast, intracellular ROS in the apoptotic cells could not further enhance the JNK signal (Supplementary Figure S3E). This result is consistent with our earlier data (Figures 2C and D). Therefore, secondary apoptosis around the primary necrotic core may slow down the spreading necrotic insults (Figure 6F).

Requirement of the Eiger/ROS/JNK signaling in cell death induced by hypoxia or oxidative stress. In addition to calcium overloading, neuronal death can be triggered by hypoxia and oxidative stress during ischemic stroke in mammals. Therefore, we ask whether the Eiger/ROS/JNK signaling is also required for cell death-induced by hypoxia and oxidative stress in Drosophila. To induce hypoxia-mediated death, the third instar larva was immersed in the Drosophila hemolymph HL3 saline. Twenty hours after hypoxia, ROS levels were greatly elevated in the eye discs (Supplementary Figures S4Aa). The result showed that overexpression of catalase and GTPx-I or LOF of Eiger and JNK significantly diminished the ROS elevation (Supplementary Figures S4Ab–d). For oxidative stress, we
treated the larval eye discs with ectopically applied H$_2$O$_2$ (0.003%, g/ml) in tissue culture. After 10 h of treatment, AO staining was significantly increased (Figure 6Aa). Functionally, GOF constructs for catalase and GTPx-1 (GMR > catalase/GTPx-1), LOF eiger (GMR > eiger RNAi) or hep (hep$^+$) all rescued the cell death (Figures 6Ab–e). Together, these results suggest that the Eiger/ROS/JNK signaling pathway is required for hypoxia and oxidative stress-induced cell death in Drosophila.

### The role of extracellular ROS in eiger expression and release

Reducing JNK signaling could rescue the cell death in the Drosophila eye disc under H$_2$O$_2$ stress. Consistently, we also found JNK activation when treating eye discs expressing a JNK reporter, puc-lacZ, with H$_2$O$_2$ (Figures 6Ba and b). Interestingly, GMR > eiger RNAi could suppress the JNK activation, suggesting that eiger works downstream of H$_2$O$_2$ (Figure 6Bc). Mechanistically, H$_2$O$_2$ treatment could increase transcript and protein levels of Eiger (Figures 6C and D). In addition, H$_2$O$_2$ may enhance Eiger release (Figure 6E). Taken together, extracellular ROS may activate JNK through Eiger by promoting the latter’s transcription and release.

In addition to the activation of JNK, we observed that overexpression of Eiger in the sev > eiger flies could suppress the JNK signal at basal conditions or after H$_2$O$_2$ treatment (Supplementary Figure S4). These results indicate that low levels of Eiger GOF may antagonize JNK signaling.

Consistently, TNF$^+$ pretreatment has been shown to be neuroprotective in the mammalian brain after stroke, likely through activation of ceramide, a second messenger involved in multiple functions.$^{28}$

### Genetic screen for modifiers of sev > GluR1$^{LC}$

To identify dominant suppressors for sev > GluR1$^{LC}$, we performed a genome-wide screen using a deficiency kit. We found 23 strong suppressors from nearly 400 deficiency lines that cover most of the genome (Table 1). As positive controls, three deficiency lines disrupting the IAP antagonist and JNKK were identified. Previously, mutations in the metabolic energy production pathways have been identified as strong suppressors from nearly 400 deficiency lines that may be shared with that screen (Table 1). Consistently, we identified five deficiency lines (three metabolic mutants and two undefined lines) that may be shared with this screen (Table 1).

We also performed a GOF screen using ~3400 EPgy2 lines$^{29}$ and an LOF screen using nearly 1000 TRiP RNAi lines.$^{30}$ From these screens, we identified 15 strong suppressors (9 for EPgy2 and 6 for RNAi). Together, seven functional groups were identified, including genes functioning in caspase-dependent apoptosis, JNK signaling and metabolism, oxidation-reduction, trafficking, ubiquitination, nuclear export and synapse activity (Table 1). Because the EPgy2 and RNAi lines are based on the UAS/Gal4 system, the modifiers from these two screens should mainly affect primary necrosis. In theory, they may also affect the spreading factors, such as Eiger or ROS.
**Extracellular ROS**

**Caspase-mediated Apoptosis**

**JNK-mediated apoptosis**

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**Prevent further spreading death**

**Primary necrosis**

**Spreading apoptosis**

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**Other apoptosis 45%**

**47%**

**Intracellular ROS**

**JNK-mediated apoptosis**

**Caspase-mediated apoptosis**

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**8%**

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Figure 6  Eiger/ROS/JNK signaling in oxidative stress. (A) Eiger/ROS/JNK signaling is required for cell death induced by 0.03% H$_2$O$_2$ in larval eye disc. The OA staining patterns were shown in a–d. Their bright field images were shown in the lower panel (a1–d1). (a) H$_2$O$_2$ could induce cell death, as determined by AO staining. (b–d) The cell death induced by H$_2$O$_2$ could be suppressed by eigerRNAi or overexpression of catalase, GTPx-1 or hep1 mutant. The ratio of positive AO spots in a fixed area (every confocal 63/C23 field, 6684.04 mm$^2$) relative to the control is shown in the right panel. (B) Eiger is required for JNK activation by 0.03% H$_2$O$_2$ in larval eye disc. (a and b) Ectopically applied H$_2$O$_2$ activates JNK signaling. (c) H$_2$O$_2$ treatment could not activate JNK signaling (indicated by lacZ staining) in the eye disc of GMR4eigerRNAi flies. (C) Real-time PCR for eiger transcripts. Trial n = 3. The result showed that H$_2$O$_2$ treatment activated eiger transcripts. (D) Immunostaining by anti-Eiger in the larval eye disc of w1118 flies. The result shows that the protein level of Eiger is higher after H$_2$O$_2$ treatment. (E) Immunostaining by anti-Eiger and anti-GFP. The data showed that H$_2$O$_2$ treatment disrupted plasma membrane of the cells and more Eiger distributed into the extracellular space. (F) Model of spreading death in the sev>GluR1Lc flies. Primary necrotic neurons release ROS and Eiger to spread death. The ratio of different spreading apoptosis is shown. For JNK-mediated death, extracellular ROS triggers expression and release of Eiger, which activates JNK and intracellular ROS. Intracellular ROS cannot further activate JNK signaling and therefore terminates spreading death by apoptosis.
Opposing effects of metabolic pathways on primary necrosis and spreading apoptosis. For the class 2 genes, we observed apparently contradictory results. The two metabolic point mutations (wal^{P2516} and cyt-c-{	extsuperscript{d-bn-1}}) appeared as suppressors, while the RNAi lines appeared as enhancers (Figures 7Aa–g; Table 1). Because their effects...
were unaltered in the GMR-P35; sev > GluR1<sup>LC</sup> flies, these genes should not affect caspase-dependent apoptosis (Figures 7Aa1–g1). We found that these genes have opposing effects on primary necrosis and Eiger-mediated apoptosis; their LOF suppressed Eiger-mediated apoptosis (Figure 7B) and enhanced primary necrosis (Figures 7C and D).

**Regulation of neuronal necrosis and spreading death by diverse family of proteins.** From these screens, gene functions in trafficking (class 4) were enriched, including three genes in the small GTPase pathway (Rho1, Rho-associated kinase (ROCK) and sqh) and two genes in another small GTPase pathway (rab8 and rab9) (Figures 7Ea–d). As a key regulator of cytoskeleton and cell polarity, the small GTPase pathway has been suggested to be involved in a wide range of diseases, including stroke and Alzheimer’s disease. We verified that ROCK<sup>RNAi</sup> and sqh<sup>RNAi</sup> indeed rescued primary necrosis in the larval eye disc of the sev > GluR1<sup>LC</sup> flies (Figure 7Ee–g and e1–g1). In addition, GOF β’Cop, another trafficking gene, rescued primary necrosis (Figure 7Eh).

We also identified genes functioning in ubiquitination, nuclear transport and synaptic structure (Table 1, class 5–7). For the ubiquitin pathway, we identified cul-2, cul-3, Ubi-p63E and Cct5. These genes may involve in proteasomal function, protein degradation and neuromuscular junction development. We also identified three genes related to synaptic activities, including lbm, Nsf2 and wnt2 (Table 1). These genes are required for synapse assembly, synaptic transmission or protein localization in the neuromuscular junction.

From these screens, we conclude that neuronal necrosis and spreading death involve distinct gene functions and cellular events.

**Discussion**

Our model provides a genetic system of neuronal necrosis and its spreading death in the *Drosophila* eye. More broadly, this model may improve our understanding of the complicated molecular and cellular events in stroke, especially primary necrosis, necrosis-mediated spreading death, and interactions between necrotic neurons and their adjacent cells.

Furthermore, our screen had identified proteins that function in multiple pathways, such as the ubiquitin proteasomal pathway, nuclear export and synaptic formation, all of which have been implicated in ischemic stroke. In general, neuronal necrosis has been considered as an irreversible process because of the severe organelle damage. Our screening result suggests that this damage process may involve multiple distinct genetic pathways. Therefore, it is possible to target necrotic progression.

For the mechanism of spreading death, our data demonstrate that different cell types may use different strategies in response to spreading insults. For instance, activation of JNK in neurons may trigger caspase-independent apoptosis, likely through Eiger. Activation of hid in non-neuronal cells may promote caspase-dependent apoptosis. It is possible that the hid pathway is also mediated by Eiger. Moreover, other unknown death pathways may also trigger neuronal apoptosis. Further, using our simplified genetic model, we found that manipulating ROS and Eiger in GluR1<sup>LC</sup>-expressing neurons could affect the JNK-dependent death and the eye defect without affecting the primary necrosis. Therefore, the spreading death is initiated by ROS and Eiger from the primary necrotic neurons.

Necrotic cells can induce apoptosis by releasing toxic factors. We propose that apoptosis may terminate the necrotic insults for the following reasons. First, caspase-dependent apoptosis induced by sev~pr~ could not spread death. Second, TNF<sub>z</sub>/JNK/ROS-mediated death could not further spread death because ROS generated in the JNK-dependent apoptosis could not further activate JNK signaling. Regarding TNF<sub>z</sub>/JNK/ROS signaling, some researchers have proposed that a positive feedback loop from TNF to JNK and then to ROS may enhance cell death because elevated ROS can activate JNK signaling and associate with TNF<sub>z</sub> and JNK-mediated death. Our data indicate that such a feedback loop does not exist during necrosis-mediated spreading death in *Drosophila*.

Regarding the translational applications of cell protection, our data suggest targeting several pathways, including neuronal necrosis, caspase-dependent and -independent death-spreading pathways, and likely other undefined pathways. Our data suggest that chelating ROS may be beneficial. Interestingly, edaravone, an ROS scavenger drug, has been tested in clinical trials for the treatment of ischemic stroke. Targeting TNF<sub>z</sub> may also be desirable because it is critical for triggering JNK-mediated apoptosis. Recently, targeting TNF<sub>z</sub> with an engineered chimeric monoclonal antibody that could pass the blood–brain barrier has been shown to be neuroprotective in a mouse model of stroke. Another advantage of targeting TNF<sub>z</sub> is that reducing its level may activate its preconditioning function. Our genetic modeling is limited by the fact that necrosis induced by the leaky channel may not reflect physiological conditions in human disease. Further investigations are required to determine whether the proteins identified here are suitable drug targets.

**Materials and Methods**

**Fly maintenance and stocks.** *Drosophila* stocks were raised on standard sucrose/cornmeal medium at constant 25 °C with a 12-h light/dark cycle. The following *Drosophila* strains are kind gifts from various laboratories: *puc<sup>E69</sup>-lacZ* and *UAS-eiger<sup>RNAi</sup>* (Dr. Tian Xu); *Al<sup>PO</sup>* (Dr. Josef Penninger); *UAS-IAP2* tested in clinical trials for the treatment of ischemic stroke. Targeting TNF<sub>z</sub> may also be desirable because it is critical for triggering JNK-mediated apoptosis. Recently, targeting TNF<sub>z</sub> with an engineered chimeric monoclonal antibody that could pass the blood–brain barrier has been shown to be neuroprotective in a mouse model of stroke. Another advantage of targeting TNF<sub>z</sub> is that reducing its level may activate its preconditioning function. Our genetic modeling is limited by the fact that necrosis induced by the leaky channel may not reflect physiological conditions in human disease. Further investigations are required to determine whether the proteins identified here are suitable drug targets.
The early third instar larvae of (ab9361; Abcam, Eugene, OR, USA), mouse anti-
USA), anti-GluR1 (ab 32132; Abcam, Eugene, OR, USA), chicken anti-
Eugene, OR, USA), anti-cleaved caspase 3 (9661; Cell Signaling, Danvers, MA,
Dcp-1Prev/Dcp-1Prev, drICE Delta1/Tb
TMRM and lacZ staining were performed as described. 22,47–50 For the PI (P4170;
Histology of larval tissue staining.
procedures followed a standard protocol.
30 s at 80
Histology in adult eyes.
Dr. Michisuke Yuzaki) and subcloned into the pUAST vector.
was generated in a w1118
Invitrogen Molecular Probe, Eugene, OR, USA) in Schneider’s medium at 25
hypoxia, we dissected the eye disc and stained them with DHE.
Normally, these larvae recovered within 15 min.
The third instar larvae were incubated in a
1.5-ml tube with HL3 saline for 45 min. Then, the larvae were returned to normal
intensity at 510 nm excited by 340 or 380 nm was calculated as the relative
m
Ru486 (mifepristone M8046; Sigma) for 90 s. 51 After 5 h of induction, we
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Conflict of Interest
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