Ras-like Gem GTPase induced by Npas4 promotes activity-dependent neuronal tolerance for ischemic stroke

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Ischemic stroke, which results in loss of neurological function, initiates a complex cascade of pathological events in the brain, largely driven by excitotoxic Ca\(^{2+}\) influx in neurons. This leads to cortical spreading depolarization, which induces expression of genes involved in both neuronal death and survival; yet, the functions of these genes remain poorly understood. Here, we profiled gene expression changes that are common to ischemia (modeled by middle cerebral artery occlusion [MCAO]) and to experience-dependent activation (modeled by exposure to an enriched environment [EE]), which also induces Ca\(^{2+}\) transients that trigger transcriptional programs. We found that the activity-dependent transcription factor Npas4 was up-regulated under MCAO and EE conditions and that transient activation of cortical neurons in the healthy brain by the EE decreased cell death after stroke. Furthermore, both MCAO in vivo and oxygen-glucose deprivation in vitro revealed that Npas4 is necessary and sufficient for neuroprotection. We also found that this protection involves the inhibition of L-type voltage-gated Ca\(^{2+}\) channels (VGCCs). Next, our systematic search for Npas4-downstream genes identified Gem, which encodes a Ras-related small GTPase that mediates neuroprotective effects of Npas4. Gem suppresses the membrane localization of L-type VGCCs to inhibit excess Ca\(^{2+}\) influx, thereby protecting neurons from excitotoxic death after in vitro and in vivo ischemia. Collectively, our findings indicate that Gem expression via Npas4 is necessary and sufficient to promote neuroprotection in the injured brain. Importantly, Gem is also induced in human cerebral organoids cultured under an ischemic condition, revealing Gem as a new target for drug discovery.

Significance

Stroke is the second leading cause of death and the most frequent cause of disability in adults worldwide (1). Ischemic stroke initiates a complicated and highly interdigitated cascade of pathological events (including excitotoxicity, oxidative stress, inflammation, and apoptosis) and results in cellular damage and loss of neurological function (2–5). Because of the high energy demands of the brain, neurons are immediately depleted of energy by any impedence of cerebral blood flow, resulting in loss of resting membrane potential and uncontrolled glutamate release (3, 6). These insults trigger repetitive depolarization, called spreading depolarization, in neurons within the infarct area (6), leading to increased intracellular Ca\(^{2+}\) levels, production of inflammatory cytokines and growth factors, and transcription of immediate early genes (2, 4, 7). These ischemia-induced genes can activate both the neuroprotective program and pathogenic cascades, which culminate in apoptotic or necrotic cell death (2, 8). The observation that both protective and pathogenic cascades are coactivated by ischemia suggests that potentiation of protective signaling pathways may block the cytotoxic effects of ischemia; however, it remains unclear how to potentiate the neuroprotective program that is characteristic of ischemic responses.

In the healthy brain, external sensory stimulation, including an enriched environment (EE), induces neuronal Ca\(^{2+}\) transients and gene expression, leading to synaptic plasticity in neurons for learning and memory (9, 10). Furthermore, nuclear Ca\(^{2+}\)/calmodulin signaling controls expression of neuroprotective genes in the healthy brain (11, 12). However, it remains unclear 1) how neuronal activity–regulated genes in the healthy brain are different from ischemia-regulated genes in the pathological brain and 2) whether induction of neuronal activity–regulated genes in the healthy brain affects neuroprotection after stroke. In this study, we found that transient activation of cortical neurons in the healthy brain decreases cell death after stroke (i.e., via activity-dependent ischemic tolerance). On the basis of a systematic search, we identified the activity-dependent transcription factor Npas4 (neural PAS domain protein 4), which is necessary for neurons to resist ischemia. Although Npas4 plays a neuroprotective role in ischemic stroke (13–15), it is unclear how it regulates this process at a molecular level. On the basis of another systematic search, we
identified a molecule, Gem, which acts downstream of Npas4 and mediates neuroprotection; these findings point to a good therapeutic target for stroke.

Results

A Short Exposure to an EE Facilitates Neuroprotection after Stroke. Rats housed in an enriched cage (provided with ladders, tubes, hiding places, etc.) for 1 mo show better functional outcomes after stroke than those housed under standard conditions (16). However, it remains unknown how an EE facilitates neuroprotection. To understand the relationship between EE and stroke, we performed middle cerebral artery occlusion (MCAO) in mice preexposed to an EE for 40 min (Fig. 1A). Intriguingly, even this short period of exposure to an EE was sufficient to decrease infarct volume (48.3 ± 9.8%) compared with that in the control group in the home cage (Fig. 1B and C). However, imposing a 6-h interval between the EE exposure and MCAO surgery prevented this protective effect. To confirm the requirement of neural activity for neuroprotection, we utilized the Syn–TetOff system (17) with an adeno-associated virus (AAV) vector carrying the excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) gene, hM3Dq-mCherry, to be expressed in neocortical neurons (Fig. 1D–F). Chemogenetic activation with an injection of clozapine N-oxide (CNO) induced Fos (activity-dependent gene) expression in these cortical neurons (Fig. 1G and SI Appendix, Fig. S1D). As expected, CNO injection at 40 min before MCAO significantly reduced the infarct volume (46.8 ± 11.0%) compared with that in the control, whereas CNO administered after MCAO did not (Fig. 1H). These results suggest that neural activation with either EE or chemogenetics before stroke is sufficient to protect neurons from ischemic death (activity-dependent ischemic tolerance).

Search for Genes Whose Expression Is Altered by Stroke and EE. Preexposure to a brief period of ischemia (brief ischemia) induces a neuroprotective mechanism in which neurons acquire tolerance to ischemia (termed ischemic tolerance or ischemic preconditioning) (18, 19). We hypothesized that neural depolarization under both EE and brief ischemia conditions would trigger expression of common genes underlying the protective mechanism that blocks death induced by excitotoxicity (Fig. 2B).
Therefore, we systematically searched for genes whose expression would be affected by brief ischemia as well as by the EE (Fig. 2A). First, RNA-sequencing (RNA-Seq) analysis was performed on the control and ischemic sides of the neocortex 2 h after MCAO to identify ischemia-regulated genes (SI Appendix, Fig. S2C). Brief ischemia rapidly induced gene expression in the cortex: Most of the genes (97 of the top 100) were up-regulated on the ischemic side relative to expression on the control side of the cortex (SI Appendix, Fig. S2 B and C). Second, a qRT-PCR analysis for the top 100 genes identified in this primary screen was performed using mice exposed to an EE for 40 min, indicating that 38 genes were also up-regulated (Fig. 2C–E). Among them, Npas4 was most highly expressed in neurons [neuronal nuclear (NeuN) positive] soon after ischemia (SI Appendix, Fig. S3) and was transiently induced by the EE (Figs. 2C and 3A). We confirmed that chemoattractant activation with CNO also induces Npas4 expression in cortical neurons (SI Appendix, Fig. S4 C and D).

To test the role of Npas4 in neuroprotection, we performed MCAO surgery in Npas4 knockout (KO) mice with or without EE exposure. Under non-EE conditions, infarct volumes were larger in Npas4 KO mice (155.0 ± 13.0%) than in wild-type (WT) mice (Fig. 3C), as previously reported (20). However, there was no difference in infarct volume between Npas4 KO mice exposed to EE and those kept in their home cages (Fig. 3 B and C). By contrast, Npas4 expression induced by doxycycline (Dox) administration using the AAV/Syn–TetOn system (SI Appendix, Fig. S5) significantly reduced the infarct volume after MCAO (56.6 ± 10.4%) relative to that in animals receiving phosphate-buffered saline (PBS) (Fig. 3 D–F). These in vivo studies demonstrated that Npas4 expression, induced just before stroke, is necessary and sufficient for activity-dependent ischemic tolerance.

Npas4 Expression Inhibits Excess Ca2+ Influx in Ischemic Neurons In Vitro. To identify the molecular mechanisms underlying the ability of Npas4 to protect neurons from death after stroke, we utilized an in vitro model of ischemia in which primary cultured cortical neurons (96.6 ± 0.3% MAP2+) were incubated under conditions of oxygen and glucose deprivation (OGD) (SI Appendix, Fig. S6 A–C).

![Fig. 2](https://doi.org/10.1073/pnas.2018850118)
A 1-h treatment with OGD increased transcription of *Npas4*, which peaked 1 h after OGD before returning to baseline (Fig. 4B). To reveal whether expression of *Npas4* (the human homolog) is induced in an in vitro model of human ischemia, we generated cerebral organoids from human induced pluripotent stem cells. Interestingly, *Npas4* expression was increased markedly in human cerebral organoids 1 h after OGD (250 ± 40%; Fig. 4C), suggesting that both humans and mice share a common mechanism for *Npas4* induction by ischemia. In the healthy mouse brain, *Npas4* expression is induced by Ca²⁺ signaling in a sensory experience–dependent manner (21, 22). In ischemic neurons, membrane depolarization evoked by energy depletion activates N-methyl-D-aspartic acid (NMDA) receptors and L-type voltage-gated Ca²⁺ channels (VGCCs), resulting in abnormal Ca²⁺ influx (3, 23). Consistent with this, OGD-mediated increases in both Ca²⁺ influx (*SI Appendix, Fig. S6E*) and *Npas4* expression (*SI Appendix, Fig. S6F*) in primary cortical neurons were inhibited either strongly (34.6 ± 1.9%; *SI Appendix, Fig. S6E*) (12.9 ± 2.8%; *SI Appendix, Fig. S6E*) by NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanooic acid (D-AP5) or significantly (78.2 ± 2.1%; *SI Appendix, Fig. S6E*) (66.5 ± 7.3%; *SI Appendix, Fig. S6F*) by L-type VGCC antagonist nifedipine (Nifed). The excess Ca²⁺ influx caused by OGD treatment induced death of primary neurons (24) (*SI Appendix, Fig. S6 G and H*). *Npas4* was sufficient to block this effect, as WT primary neurons transfected with AAV/C MV–*Npas4* at 24 h before OGD exhibited a decreased number of propidium iodide⁺ (PI⁺) dead cells after OGD (67.2 ± 7.0%) than those transfected with AAV/C MV–EGFP (Fig. 4D), consistent with the in vivo results (Fig. 3 D and E). Conversely, expression of *Npas4* via AAV/CMV–*Npas4* in *Npas4* KO primary neurons was sufficient to block the increase in the number of PI⁺ dead cells (Fig. 4D). These in vitro studies revealed that *Npas4* expression in primary neurons is both necessary and sufficient for protection against cell death.

Interestingly, *Npas4* overexpression before OGD suppressed an increase in Ca²⁺ influx in primary cortical neurons (79.8 ± 3.1%; Fig. 4 E and F) similar to Nifed treatment (74.9 ± 2.5%; Fig. 4F); however, it did not reduce influx further (71.5 ± 3.5%; Fig. 4F). Furthermore, *Npas4* overexpression reduced the number of dead primary neurons after excessive activation of L-type VGCCs with their agonist, Bay K8644 (BayK) (27) (77.2 ± 6.7%; Fig. 4G). These results suggest that *Npas4* expression in primary neurons might directly or indirectly inhibit L-type VGCC function. To confirm observations in vivo, WT and *Npas4* KO mice received intraperitoneal (i.p.) injections of Nifed soon after the MCAO surgery. It was reported previously that Nifed treatment at 2 d after MCAO markedly reduces infarct volume at 2 wk after MCAO (26). Consistent with this, Nifed reduced the acute infarct volume in WT mice (53.5 ± 11.6%; Fig. 4I). Intriguingly, the infarct volumes were not different between WT and *Npas4* KO mice treated with Nifed (74.1 ± 14.6%; Fig. 4 H and I), although under the control conditions, the infarct volume was larger in *Npas4* KO mice (155.0 ± 13.0%) than in WT mice (Fig. 3C). These results strongly suggest that *Npas4* expression inhibits L-type VGCC function in vitro and in vivo.

Next, we investigated the relationship between *Npas4* expression and NMDA receptor function. As shown in *SI Appendix, Fig. S6E*, D-AP5 strongly inhibited Ca²⁺ influx (34.6 ± 1.9%) in cultured neurons during OGD to a level comparable with that under the control condition, suggesting that activation of NMDA receptors is required to induce excessive Ca²⁺ influx in ischemic neurons (*SI Appendix, Fig. S6D*), as previously reported (27). *Npas4* overexpression before OGD suppressed the increase in Ca²⁺ influx in ischemic neurons (*SI Appendix, Fig. S6D*), similar to D-AP5 treatment (28.8 ± 1.9%), but did not further reduce the Ca²⁺ influx (29.9 ± 1.5%; Fig. 4F). NMDA receptor activation enables Na⁺ and Ca²⁺ influx to further depolarize neurons (28), which in turn facilitates Ca²⁺ influx through voltage-gated Ca²⁺ channels.
Fig. 4. Npas4 expression inhibits excess Ca\(^{2+}\) influx into ischemic neurons. (A) Schematic drawing of primary cultured neurons exposed to ischemia-like OGD. (B) qRT-PCR for relative amounts of Npas4 or Fos messenger RNA (mRNA) in primary neurons after 1 h of OGD. (C) qRT-PCR for relative amounts of NPAS4 mRNA 1 h after OGD in human brain organoid cultures generated from induced pluripotent stem cells. (D) Numbers of dead cells in either Npas4-overexpressing or Npas4-deficient neurons after OGD. Primary neurons prepared from WT or Npas4 KO embryonic cortexes were transfected with AAV/CMV–EGFP or AAV/CMV–Npas4 24 h before OGD. Dead cells were stained with PI 24 h after OGD. (E) Ca\(^{2+}\) imaging during OGD in primary neurons electroporated with plasmids carrying H2B-mCherry and Npas4. Dotted circles indicate H2B-mCherry–transduced cells (E, Left). Graphs on the right indicate Fluo-4 intensities (ΔF/F\(_0\)) of Npas4-overexpressing neurons (H2B-mCherry– and Npas4-transfected; Npas4) relative to those of surrounding untransfected neurons (untransfected) during the 1-h OGD. (F) Graph shows Fluo-4 intensities of Npas4-overexpressing neurons relative to those of surrounding untransfected neurons 5 min after the onset of OGD in the presence or absence of Nifed (an inhibitor of L-type VGCCs) or D-AP5 (an inhibitor of the NMDA receptor). (G) Numbers of dead cells in Npas4-overexpressing and control neurons (transfected with AAV/CMV–EGFP and AAV/CMV–Npas4, respectively) after VGCCs were activated with BayK for 5 min. Dead cells were stained with PI 24 h after BayK treatment. (H and I) In vivo neuroprotective effects of Nifed in WT and Npas4 KO mice undergoing MCAO. Living cells were stained with TTC 24 h after MCAO (H) to calculate the infarct volume (I). (J) Schematic drawing of the deduced function for Npas4 in ischemic neurons. *P < 0.05, **P < 0.01, NS: not significant (Student’s t test in C and two-way ANOVA with post hoc Tukey’s test in D, F, G, and I).
channels (29) (SI Appendix, Fig. S6)|. Although Npas4 inhibited L-type VGCCs (Fig. 4G), it was unknown whether it would also affect NMDA receptors (Fig. 4F). However, Npas4 overexpression did not prevent the death of primary neurons induced by NMDA (100.3 ± 4.9%; SI Appendix, Fig. S7A), suggesting Npas4 does not alter NMDA receptor function in cortical neurons. To confirm these observations in vivo, mice received i.p. injections of the NMDA receptor antagonist MK801 [because D-AP5 cannot cross the blood–brain barrier (30)] soon after MCAO. As expected, MK801 administration reduced the infarct volume similarly in WT

Fig. 5. Search for Npas4 target genes conferring protection against ischemic death. (A) Schematic drawing for the search for Npas4 target genes in vitro. Genes whose expression was altered after MCAO were identified by RNA-Seq. (B) The first screen for candidate genes that were regulated in ischemic neurons in an Npas4-dependent manner. Among the top 200 genes listed in SI Appendix, Fig. S2C, expression of the 15 candidate genes was confirmed by qRT-PCR on primary cultured neurons from WT (n = 4) and Npas4 KO (n = 4) pregnant mice subjected to 1 h of OGD. (C) The second screen for the 13 differentially regulated genes listed in B. Primary neurons were cotransfected with each candidate gene and H2B-EGFP, and numbers of dead cells after OGD were counted via PI staining 24 h later. (D) Reduced numbers of dead cells overexpressing Gem in WT and Npas4 KO primary neurons after OGD. (E) Schematic drawings of Gem overexpression with the AAV/Syn-Tet3G system in primary neurons and the experimental timeline. Primary neurons from WT and Npas4 KO embryonic cortices were cotransfected with AAV/Syn–Tet3G and AAV/TRE3G–EGFP or AAV/TRE3G–Gem. After 12 h of Dox administration, primary neurons received 1 h of OGD and were subjected to PI staining 24 h later to count dead cells (D). Note that overexpression of Gem in Npas4-deficient neurons was sufficient to reduce the number of dead cells after ischemia. (F) Gem expression in cortical neurons after stroke. Two-color ISH with Npas4 (magenta) and Gem (green) was performed in the neocortex 1 h after MCAO. (G and H) The numbers of Gem+ cells (G) and the ratio of Gem+ cells to Npas4+ cells (H) in the MCAO side (MCAO) compared with those in the control side (Ctl). **P < 0.01 (Student’s t test in G and H, Student’s t test with Bonferroni correction in C and G, and two-way ANOVA with post hoc Tukey’s test in D).
and Npas4 KO mice (SI Appendix, Fig. S7 B and C). As NMDA receptor activation was required for Ca\(^{2+}\) influx in ischemic neurons (SI Appendix, Fig. S6 E), inhibition by MK801 prevented cell death in Npas4 KO mice after MCAO (SI Appendix, Fig. S7 B and C).

### Search for Npas4 Target Genes Conferring Protection against Ischemic Death

As Npas4 overexpression in primary cortical neurons did not change the expression of L-type VGCC subunits (SI Appendix, Fig. S8), it is possible that the transcription factor Npas4 controls L-type VGCC function via its downstream genes (Fig. 4 J). We therefore searched for Npas4 target genes that met the following requirements: 1) expression is regulated by ischemic stroke, 2) expression is altered by Npas4 deficiency, and 3) expression protects neurons from cell death upon infarct. Initially, ischemia-regulated genes were identified with the RNA-Seq analysis in MCAO mice (SI Appendix, Fig. S2C). Second, qRT-PCR analyses for the top 200 genes from this primary screen were performed to identify 12 genes down-regulated and 3 genes up-regulated after OGD in neurons from Npas4 KO mice compared with expression in neurons from WT mice (Fig. 5 A and B and SI Appendix, Fig. S9).

Third, to determine which genes are necessary for cell survival, we overexpressed 13 of the genes as well as Gem and an H2B-EGFP control in primary neurons via electroporation. Only two of these reduced the number of PI\(^+\) dead cells after OGD: Npas4 and Gem (Fig. 5 C). The neuroprotective effect of Gem overexpression against OGD was confirmed via the AAV/Syn–TetOn system in primary neurons from WT and Npas4 KO mice (Fig. 5 D and E), indicating that Gem overexpression is sufficient to prevent the increase in cell death of Npas4-deficient neurons (Fig. 5 D). Two-color in situ hybridization (ISH) of cortical neurons after MCAO showed that Gem expression increased significantly mainly in Npas4-expressing neurons (156.4 ± 9.0%; Fig. 5 F–H). Furthermore, in vivo experiments revealed that Npas4 overexpression with the AAV/Syn–TetOn system increased transcription of Gem and Npt2 [Npas4-downstream gene (31)] (SI Appendix, Fig. S10). These results suggest that Gem, a newly identified downstream target of Npas4, is sufficient to protect ischemic neurons from cell death.

#### Gem Expression Protects Ischemic Neurons from Death

Gem, a member of the Rem, Rad, and Gem/Kir (RGK) family of Ras-related small GTPases, regulates Ca\(^{2+}\) channel function and cytoskeletal rearrangements (32, 33). Gem expression overexpression before OGD suppressed the OGD-induced increase in Ca\(^{2+}\) influx in primary cortical neurons (75.9 ± 1.8%; Fig. 6 A). However, intracellular Ca\(^{2+}\) concentrations were not different in Gem-overexpressing and control primary neurons under normal conditions (105.7 ± 7.5%; SI Appendix, Fig. S11 B). To examine the effect of Gem on VGCC function, we performed Ca\(^{2+}\) imaging with GCaMP6f in HEK293T cells cotransfected with the genes encoding L-type VGCC subunits Cav1.2 (Cacna1c, channel) and Cav3.1 (Cacna2d3, regulatory). The cells were treated with the L-type VGCC agonist (BayK), leading to a potent increase in Ca\(^{2+}\) influx (Fig. 6 B). By contrast, cotransfection of Gem with Cav1.2 and Cav3.1 suppressed the increase in Ca\(^{2+}\) influx induced by BayK treatment (68.8 ± 1.9%; Fig. 6 B and C), while cotransfection of Npas4 along with these did not (Fig. 6 C). These observations suggested that Npas4 represses L-type VGCC function via Gem, which regulates the subcellular localization of Cav1.2 (32). In HEK293T cells cotransfected with Gem, Cav1.2, and Cav3.1, the amount of Cav1.2 protein on the cell surface was smaller (41.0 ± 10.0%) than that on HEK293T cells cotransfected with Cav1.2 and Cav3.1 but not Gem (Fig. 6 D and E).

### Discussion

The main findings of this study are that 1) a short period of neural activation with either normal stimuli (EE) or chemo- genes before stroke is sufficient to facilitate neuronal protection from ischemic death (activity-dependent ischemic tolerance); 2) Npas4 expression, induced just before stroke, is necessary and sufficient to promote activity-dependent ischemic tolerance; 3) Npas4 expression inhibits L-type VGCC function in vitro and in vivo; and 4) expression of Gem, a newly identified downstream target of Npas4, is necessary and sufficient to protect neurons from ischemic death in vitro and in vivo.

Npas4 is neuroprotective in a model of stroke (13–15). Nevertheless, it was not clear how Npas4 promotes survival of ischemic neurons after OGD in vitro and after MCAO in vivo. Synaptotagmin 10 (Syt10) (34) and mitochondrial calcium uniporter (Mcu) (35) are Npas4 downstream factors that affect neuroprotection against excitotoxicity induced by kainic acid and NMDA, respectively, in primary neurons in vitro. However, our RNA-Seq analysis of MCAO-treated mice (SI Appendix, Fig. S2) revealed that expression of Syt10 and Mcu was not altered markedly after stroke. This suggests that in the in vivo model of ischemic stroke, Npas4 might regulate unknown targets to protect ischemic neurons from cell death. In this study, we identified Gem as an Npas4 target, which is necessary and sufficient to protect neurons from cell death after in vitro and in vivo ischemia (Fig. 6).

It is well known that a patient who either had a recent transient ischemic attack or recovered from a mild stroke is at high risk of recurrence (36). Although pretreatment with brief ischemia induces a neuroprotective mechanism (18, 19), it is difficult to apply this to patients. Interestingly, animal experiments reveal that exercise preconditioning (walking on a treadmill) provides significant neuroprotection against stroke (37), although at least 2 or 3 wk of pretraining is necessary to induce ischemic tolerance. By contrast, our mouse ischemic models revealed that a short period of neural activation before stroke is sufficient to acquire ischemic tolerance (Fig. 1). As shown in the results from the DREADD system (Fig. 1 H), the method that artificially modulates the brain state may lead to a new therapy for stroke. Furthermore, our results suggest that transient induction of activity-regulated genes in the healthy brain activates a neuroprotective mechanism and facilitates cell survival after stroke (Fig. 1). Consistent with this, our systematic searches for such responsible genes revealed that Npas4 and thus Gem play a central role in activity-dependent ischemic tolerance (Figs. 2 and 5). The number of Npas4\(^{+}\) cells in mice overexpressing Npas4 by

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**Fig. 6.** Gem expression protects ischemic neurons from death. (A) Ca^{2+} imaging during OGD in primary neurons electroporated with plasmids carrying H2B-mCherry and Gem. Graph shows Flu-4 intensities of Gem-overexpressing neurons (Gem) relative to those of surrounding untransfected neurons (untransfected) 5 min after the onset of OGD. (B) Ca^{2+} imaging of GCaMP6f-transfected HEK293T cells during treatment with BayK (VGCC agonist). GCaMP intensities were measured in HEK293T cells expressing GCaMP6f, VGCC subunits (Cav1.2 and Cav3), and Gem during BayK treatment. (C) Graph showing relative GCaMP intensities 3 min after Bayk treatment (arrow in B). (D) Western blot for Cav1.2 protein on the cell surfaces of HEK293T cells expressing VGCC subunits (Cav1.2 and Cav3) and either Gem or Npas4. Graph shows amounts of the cell surface Cav1.2 protein relative to the protein amounts in the cell lysates. (E) Western blot for endogenous Cav1.2 protein in primary neurons in which Npas4 or Gem was overexpressed. Graph shows amounts of the cell surface Cav1.2 protein relative to the amounts in cell lysates. (F) Gem overexpression via the AAV/Syn–TetOn system in the cortex in vivo. AAV/Syn–Tet3G and AAV/TRE3G–Gem were coinjected into the lateral ventricles of postnatal day 0 (P0) pups. qRT-PCR was performed to calculate relative amounts of each messenger RNA (mRNA) after PBS or Dox treatment. (G) Effects of Gem overexpression with the AAV/Syn–TetOn system before MCAO. AAV/Syn–Tet3G and AAV/TRE3G–Gem were coinjected into the lateral ventricles of WT and Npas4 KO pups at P0; 42 d later, the mice received PBS or Dox treatment 24 h before MCAO. Living cells were stained with TTC 24 h after MCAO to calculate the infarct volume. (H) Living cells in WT and Gem KO mice were stained with TTC 24 h after MCAO to calculate the infarct volume. Note that preexposure to an EE is not neuroprotective against ischemia in Gem KO mice. (I) qRT-PCR for relative amounts of Gem mRNA 1 h after OGD in human brain organoids generated from induced pluripotent stem cells. *P < 0.05, **P < 0.01; NS: not significant (one-way or two-way ANOVA with post hoc Tukey's test in C, D, E, and H or A and G, respectively; Student's t test in F and I).

The AAV/Syn–TetOn system was larger (1,790 ± 87 cells/mm²; SI Appendix, Fig. S5) than that in mice either activated with chemogenetics or exposed to an EE (824 ± 126 and 643 ± 78 cells/mm², respectively; SI Appendix, Fig. S4). However, the rate of reduction in infarct volume after MCAO was not significantly different among these conditions (Figs. 1 and 3). Npas4 may facilitate...
neuroprotection via both cell-autonomous and non–cell-autonomous mechanisms. In the ischemic brain, abnormal depolarization of neurons induces several events that affect surrounding neurons, including uncontrolled glutamate release (3, 6), production of inflammatory cytokines (4), and cell death. It is possible that preinduction of Npas4 in a certain number of neurons before stroke may reduce Ca2+ influx via a cell-autonomous mechanism, leading to propagation to surrounding dysfunctional neurons.

Although Npas4 and Gem are reported independently as ischemia-induced genes (20, 38, 39), the functional relationship between them is unknown. Our results show that Gem is transcriptionally activated by Npas4, whose expression is induced by excessive Ca2+ influx into the cytoplasm of neurons after stroke (Fig. 6). Gem suppresses localization of the L-type VGCC to the cytoplasmic membrane, leading to inhibition of excess Ca2+ influx and thereby protecting neurons from excitotoxic death. These findings are consistent with the protection conferred by Npas4 against seizure-induced damage in hippocampal neurons (12). Transcription of Gem, as well as the related RKG family member Rem2, is up-regulated by extracellular stimuli (40, 41). Actually, we found that both EE and brief ischemia induce Gem expression (Fig. 2 and SI Appendix, Fig. S2). Rem2 inhibits VGCC currents, promotes development of excitatory and inhibitory synapses, and is involved in dendritic branching (33, 41, 42), whereby it controls neural plasticity in the visual cortex (43). Intriguingly, Gem positively regulates dendritic branching in an activity-dependent manner (40). Npas4 also regulates dendritic spine formation and controls the excitatory–inhibitory balance within neural circuits (21, 31, 44). In the healthy brain, the neural activity–evoked Npas4 increases both inhibitory synapses in excitatory projection neurons and excitatory synapses in inhibitory interneurons (31), enabling neurons to calm down. This raises the possibility that expression of Gem via Npas4 is part of a feedback loop that controls Ca2+ influx into neurons to regulate neuropotency in the healthy brain and facilitate neuroprotection in the injured brain. Remarkably, expression of Npas4 and Gem increased not only in mouse brain but also human cerebral organoids after ischemic treatment (Figs. 4C and 6f). Therefore, it is possible that Gem generally functions downstream of Npas4 in various brain areas, nominating it as a good target for drug discovery aimed at neuroprotection from excitotoxicity after stroke as well as seizure.

Materials and Methods

Animal experiments were approved by the animal care committees of Nara Medical University, Kagawa University, and Osaka University in accordance with the policies established in the NIH Guide for the Care and Use of Laboratory Animals (45). Details of materials regarding a list of animals, plasmds, primers for RNA-Seq analysis, and antibodies used for our study can be found in SI Appendix. Furthermore, methods detailing brain surgery techniques, primary neuronal culture techniques, plasmid and adeno-associated viral vector constructions, qRT-PCR analysis, RNA-Seq analysis, immunoblot analysis, immunohistochemistry, ISH, and Ca2+ imaging techniques can also be found in SI Appendix.

Data Availability. All study data are included in the article and/or supporting information.

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