Targeted disruption of Kv2.1-VAPA association provides neuroprotection against ischemic stroke in mice by declustering Kv2.1 channels

Anthony J. Schulien1,2, Chung-Yang Yeh1,2, Bailey N. Orange1,2, Olivia J. Pav1,2, Madelynn P. Hopkins1,2, Aubin Moutal3, Rajesh Khanna3, Dandan Sun2,4, Jason A. Justice1,2*, Elias Aizenman1,2*

Kv2.1 channels mediate cell death–enabling loss of cytosolic potassium in neurons following plasma membrane insertion at somatodendritic clusters. Overexpression of the carboxyl terminus (CT) of the cognate channel Kv2.2 is neuroprotective by disrupting Kv2.1 surface clusters. Here, we define a seven–amino acid declustering domain within Kv2.2 CT (DP–2) and demonstrate its neuroprotective efficacy in a murine ischemia-reperfusion model. TAT–DP–2, a membrane-permeable derivative, induces Kv2.1 surface cluster dispersal, prevents post-injurious pro-apoptotic potassium current enhancement, and is neuroprotective in vitro by disrupting the association of Kv2.1 with VAPA. TAT–DP–2 also induces Kv2.1 cluster dispersal in vivo in mice, reducing infarct size and improving long-term neurological function following stroke. We suggest that TAT–DP–2 induces Kv2.1 declustering by disrupting Kv2.1–VAPA association and scaffolding sites required for the membrane insertion of Kv2.1 channels following injury. We present the first evidence of targeted disruption of Kv2.1–VAPA association as a neuroprotective strategy following brain ischemia.

INTRODUCTION

Stroke is the primary cause of serious long-term disability in the United States (1). Ischemic stroke is the most common form, accounting for approximately 80% of all cases, and results in focal cerebral ischemia secondary to thromboembolic arterial occlusion. While thrombolytic therapy (i.e., recombinant tissue plasminogen activator) and surgical endovascular interventions may allow for brain reperfusion following ischemia, no U.S. Food and Drug Administration–approved neuroprotective drugs are available with the capability to mitigate the irreversible neuronal cell loss commonly associated with this neurological disorder.

A hallmark of neuronal tissue damage following brain ischemia is the advent of excitotoxic injury and delayed apoptosis in the ischemic penumbra, a peri-infarct zone of collaterally perfused tissue surrounding the central necrotic core (2). A known key regulator of neuronal apoptosis is the delayed-rectifying voltage-gated potassium channel Kv2.1 (3). Although this channel normally functions to modulate cell excitability (4, 5), it also plays a critical role in neuronal demise by allowing a loss of cytoplasmic potassium, optimizing intracellular conditions for programmed cell death (3, 6, 7). Neurons with decreased functional expression of Kv2.1 are highly resistant to apoptotic stimuli (3). Kv2.1-mediated proapoptotic potassium efflux arises solely from the de novo, syntaxin-dependent insertion of a reserve pool of channels into the plasma membrane (PM) 3 to 5 hours following insult (3, 8–11), such that these apoptosis-enabling currents are mediated always and solely by new Kv2.1 channels reaching the membrane (3, 8). As a result, delayed proapoptotic Kv2.1 channel membrane insertion likely facilitates incorporation of viable penumbral tissue into the expanding infarct core. Exploration of neuroprotective strategies that mitigate delayed apoptotic cell death by inhibiting proapoptotic Kv2.1 current enhancement following cerebral ischemia are likely to address a critical clinical need to combat stroke-mediated neuronal damage.

Two separate membrane populations of Kv2.1 exist in neurons: (i) freely dispersed conducting channels, which mediate canonical delayed rectifier potassium currents that regulate neuronal excitability (4, 5, 12), and (ii) electrically silent, micrometer-sized somatodendritic channel clusters (12). This second, much larger population of clustered Kv2.1 channels forms endoplasmic reticulum–PM (ER–PM) junctions that function as scaffolding sites for ion channel trafficking to the membrane (13), facilitating surface delivery of new proapoptotic Kv2.1 channels (14, 15). Critically, overexpression of the C terminus of the cognate channel Kv2.2 (Kv2.2 CT) can disrupt Kv2.1 clusters without altering the biophysical properties of existing active channels (14, 16) and, importantly, block proapoptotic potassium channel current enhancement, resulting in neuroprotection following oxidative insult (14). Here, we identify a critical seven–amino acid sequence within the Kv2.2 CT responsible for mediating Kv2.1 cluster dispersal and generate an injectable, blood-brain barrier–permeant, neuroprotective therapeutic peptide (TAT–DP–2) based on this sequence. We show that this drug provides robust neuroprotection in vitro and, most critically, provide the first evidence of targeted disruption of Kv2.1 surface clusters as a neuroprotective strategy in vivo by showing that TAT–DP–2 administration following ischemic stroke provides robust neuroprotection in mice. As recent evidence suggests that the ER vesicle-associated membrane protein–associated protein A (VAPA) recruits Kv2.1 to the clusters (17–19), we demonstrate that this peptide disrupts this interaction, without directly binding VAPA, successfully translating this molecular finding into a viable clinical strategy.
RESULTS

Identification and characterization of a Kv2.1 declustering peptide

We first defined the minimal sequence within Kv2.2 CT that mediates Kv2.1 declustering (16) and neuroprotection (14). To accomplish this, we analyzed the CT domains of both Kv2.2 (fig. S1A) and Kv2.1, focusing on the amino acid residues known to be critical for Kv2 channel surface cluster formation (20). Both Kv2.1 and Kv2.2 CT sequences contain a highly homologous segment involved in cluster formation, known as the proximal restriction and clustering (PRC) domain (20) (fig. S1B). Specifically, four critical residues exist within the PRC domain of Kv2.1 (S587, S590, F591, and S593; rat sequence, UniProt P15387) that, when point-mutated, abolish the ability for Kv2.1 to form clusters in neurons (20). A seven–amino acid sequence that includes these four residues from Kv2.1 (amino acids 587 to 593; SIDSFIS) differs only by a single amino acid in the analogous segment of Kv2.2 CT (amino acids 602 to 608; SIDSFTS). Notably, these sequences are also conserved for both Kv2.1 and Kv2.2 channels in mouse (UniProt Q03717 and UniProt A6H8H5, respectively) and human (UniProt Q14721 and UniProt Q92953, respectively).

With this information, we hypothesized that the region encompassing amino acids 602 to 608 within Kv2.2 CT was most likely to be responsible for disrupting Kv2.1 clusters, likely by outcompeting a “cluster targeting” sequence on existing channels that recruited them to the cluster microdomain. Kv2.1 and Kv2.2 target somatodendritic clusters via noncanonical two phenylalanine in an acidic tract (FFAT) motif sequences, namely SFISCAT and SFTSCAT (17–19), which overlap substantially with our proposed domain (fig. S1B). These sequences interact with the ER linker VAPs (17, 19). As these sequences omit one critical residue strongly implicated in cluster formation (S602 in Kv2.2 and S587 in Kv2.1), we opted to evaluate a sequence that includes all four critical proclustering residues (fig. S1C), generating the declustering peptide, DP-2 (SIDSFTS), and its scrambled control, Sc-2 (DFSSIST).

We also generated plasmids encoding the DP-2 and Sc-2 sequences for comparative purposes, as prior work had used a Kv2.2 CT–expressing vector to decluster Kv2.1 channels (12, 14). These expression vectors include the native upstream sequence containing the most proximal methionine to act as the start codon. When expressed, the putative declustering peptide (DP-1) and its scrambled control (Sc-1) are MKSTSSIDSFTS and MKSTSDFSSIST, respectively (fig. 1A and fig. S1D). These were tested first for their declustering properties by transfecting primary cortical neurons with a green fluorescent protein (GFP)–tagged Kv2.1 construct, which produces somatodendritic Kv2.1 channel clusters similar, albeit not identical, to endogenous channels (21). Neurons were first cotransfected with either empty vector (pcDNA3), Kv2.2 CT–expressing plasmid (pCMV-Kv2.2CT), DP-1–expressing plasmid (pCMV-DP-1), or Sc-1–expressing plasmid (pCMV-Sc-1). Twenty-four hours following transfection, Kv2.1 cluster densities were analyzed. When compared to empty vector–expressing or Sc-1–expressing neurons, we found that both Kv2.2 CT and DP-1 expression similarly resulted in significant Kv2.1 cluster dispersal (Fig. 1, B and C). These results suggest that a short sequence within Kv2.2 CT, contained within the DP-1 peptide, is sufficient for dispersal of Kv2.1 surface clusters in vitro.

To test the hypothesis that SIDSFTS (DP-2) was the minimal sequence responsible for Kv2.1 declustering, we generated a cell-permeant, transactivator of transcription (TAT)–linked derivative of DP-2 (TAT-DP-2), as well as a scramble control (TAT-Sc-2; fig. S1C). TAT (YGRKKRRQRRR) is a cell-penetrating peptide derived from HIV-1 (22). To assess the ability of TAT-DP-2 treatment to...
induce rapid dispersal of Kv2.1 clusters, we again cotransfected cortical neurons with a GFP-tagged Kv2.1 construct. Twenty-four hours following transfection, neurons were treated with either TAT-DP-2, TAT-Sc-2, or vehicle for 2 hours. We found that TAT-DP-2 (10 μM) induced a time-dependent (Fig. S2, A and B) dispersal of Kv2.1 surface clusters, with significant declustering observed at 2 hours when compared with either vehicle or TAT-Sc-2 (10 μM) treatment (Fig. 2, A and B). Lower concentrations of TAT-DP-2 (3 μM) induced significant dispersal of Kv2.1 surface clusters, but only when compared to vehicle-treated neurons. Hence, we hypothesize that TAT-DP-2 is an effective in vitro declustering tool at concentrations between 3 and 10 μM. Kv2.1 clusters appear to remain dispersed up to 24 hours following initial treatment with TAT-DP-2, although to a lesser degree, indicating that this effect is likely reversible (Fig. S3). The ability of TAT-DP-2 to decluster endogenous Kv2.1 channels in cortical neurons in vitro was confirmed by immunocytochemistry (Fig. S4).

**DP-2 alone does not bind to VAPA but effectively disrupts Kv2.1-VAPA association**

Kv2.1 and Kv2.2 channels are recruited to ER-PM junctions via CT PRC-mediated interaction with VAPA/B (17–19). As indicated earlier, this process occurs at noncanonical FFAT binding motifs within the PRC domains of Kv2.1 and Kv2.2 (Fig. S1B), which are recruited to the FFAT binding domain on VAPA/B (17–19). We used a peptide spot array assay (23) to assess the ability of DP-2, along with many mutant variants of the peptide (see below; fig. S5A), to bind VAPA in a cell-free assay. Unexpectedly, despite the fact that noncanonical FFAT motifs SFISCAT and SFTSCAT are required for Kv2.1 and Kv2.2 interaction with VAP proteins, we observed no binding between these peptides and VAPA (Fig. S5A). Moreover, we observed no binding between VAPA and DP-2. These data indicate that both the DP-2 sequence and the noncanonical FFAT motifs located on Kv2.1 and Kv2.2 may not be sufficient for VAPA binding alone, at least in a cell-free environment. This assay was validated by observing strong VAPA binding to the positive control peptide (EFFDAPE), a canonical FFAT motif.

Given these results, we were thus interested in testing whether a TAT-linked derivative of the canonical FFAT motif (TAT-EFFDAPE) could disrupt Kv2.1 surface cluster formation. GFP-Kv2.1–transfected neurons were treated with TAT-EFFDAPE (TAT-FFAT; 10 μM) or a scramble control peptide TAT-FDEEFAP (TAT-FFATSc; 10 μM) for either 2 or 24 hours and analyzed for Kv2.1 cluster density. As TAT-FFAT treatment did not induce Kv2.1 cluster dispersal (Fig. S5, B and C) when compared with a scrambled sequence peptide, our results suggest that a more complex interaction between VAPA and Kv2.1 may be at play.

Selective serine phosphorylation of Kv2.1 within its PRC domain appears critical for its recruitment by VAP proteins, most notably S90 (S605 on Kv2.2) (17–19). CD4-linked chimeras of Kv2.1 PRC domain peptides localize to VAPA proteins at the ER, an effect abolished by selective serine-to-alanine mutations within the PRC domain (19). Conversely, mutation of these same residues to aspartic acid restores CD4-linked PRC-VAPA interaction (19). Although these phosphorylation events had been proposed to render the PRC domain as containing noncanonical FFAT motifs (17–19), we were also unable to observe demonstrable binding to VAPA with these amino acids sequences in the peptide array. The pseudo-phosphorylated mutant DP-2 peptide sequences lacked VAPA binding capacity in our peptide spot array assay (Fig. S5A). Regardless of the observed lack of VAPA binding, a TAT-linked peptide containing serine-to-aspartate mutations (TAT-DP-2D:TAT-DIDDFTD) effectively declustered Kv2.1-GFP aggregates in neurons, while the serine-to-alanine mutant (TAT-DP-2A:TAT-AIDFTA) did not (Fig. S6, A and B).
Together, these results strongly indicate that TAT-DP-2–mediated Kv2.1 declustering relies on phosphorylation of serine residues, although this sequence alone is not sufficient to bind to VAPA (fig. S5A), implicating a multifaceted architecture underlying the interaction between Kv2.1 and its associated ER binding partner.

Despite the lack of demonstrable direct binding between TAT-DP-2 and VAPA, we evaluated whether TAT-DP-2 could still disrupt the Kv2.1–VAPA interaction in vitro, given its strong declustering effect. We used a proximity ligation assay (PLA; Fig. 3A), which allows for analysis of protein-protein association at distances less than 40 nm from each other (24). We treated cortical neurons with vehicle, TAT-Sc-2 (10 μM), or TAT-DP-2 (10 μM) for 2 hours. Following treatment, we used a PLA staining protocol that allowed for fluorescent labeling of Kv2.1–VAPA association based on published methods (24). We observed robust fluorescent PLA puncta formation in both vehicle and TAT-Sc-2–treated neurons (Fig. 3, B and C), indicating a high level of Kv2.1–VAPA association. In contrast, TAT-DP-2 exposure induced a significant reduction in PLA reactions (Fig. 3, B and C), demonstrating that this peptide alone could effectively disrupt Kv2.1–VAPA association, seemingly without directly binding to VAPA.

**TAT-DP-2 prevents enhancement of Kv2.1–mediated proapoptotic potassium currents and is neuroprotective in vitro**

The cell death–enabling loss of intracellular potassium in neurons can be experimentally monitored as a time-dependent enhancement in Kv2.1-mediated potassium currents under whole-cell voltage clamp conditions as new channels become incorporated into the PM (3, 8, 25). To evaluate whether TAT-DP-2–induced declustering could prevent the apoptotic enhancement of potassium currents in a manner akin to Kv2.2 CT overexpression (14), we treated cortical neurons in vitro with either TAT-DP-2 (10 μM), TAT-Sc-2 (10 μM), or vehicle for 2 hours before a 2-hour exposure to 60 mM threo-β-benzylxystarptic acid (TBOA; 60 μM) or vehicle. TBOA is a glutamate transporter inhibitor that produces a slow excitotoxic injury via prolonged N-methyl-D-aspartate (NMDA) receptor stimulation (26). This induces a pronounced potassium current surge mediated by Kv2.1, culminating in delayed apoptotic cell death, mimicking the excitotoxic conditions present in the ischemic penumbra following stroke (9, 11, 14, 26).

Delayed rectifier potassium currents, which are primarily mediated by Kv2.1 in our preparation (3), were monitored 3 to 5 hours following TBOA exposure. As expected, Kv2.1 current densities were significantly increased in the vehicle/TBOA-treated cells when compared with the vehicle/vehicle-treated group (Fig. 4, A and B). An identical effect was also seen when comparing the TAT-Sc-2/vehicle–treated group with the TAT-Sc-2/vehicle–treated group. Notably, treatment with TAT-DP-2 completely abolished TBOA-mediated enhancement of Kv2.1 current densities, as no differences were noted between TAT-DP-2/vehicle–treated neurons and TAT-DP-2/TBOA–exposed cells. These results support the notion that DP-2–mediated Kv2.1 declustering can effectively prevent the apoptotic surge of potassium currents in neurons by disrupting their membrane insertion platform. Note that baseline Kv2.1 current densities were unaffected by the peptide treatment, demonstrating that the injury-mediated insertion of Kv2.1 channels and the normal trafficking of the channel occur via distinct processes, as we have repeatedly experimentally ascertained in prior works (3, 8, 9).

With evidence that TAT-DP-2–mediated declustering prevents enhancement of proapoptotic Kv2.1 potassium currents following injury, we next tested the neuroprotective actions of TAT-DP-2 in cortical neurons in vitro. Cells were exposed to either vehicle, TAT-DP-2 (3 μM), or TAT-Sc-2 (3 μM) for 2 hours before an overnight exposure to either vehicle or TBOA (60 μM). In addition to the preincubation period, peptides were present for the first 4 hours of the TBOA exposure (Fig. 4C), a period of time when current enhancement normally begins following initial injury (3, 8). Neurotoxicity was assessed by the release of lactate dehydrogenase (LDH) into the culture medium, as described previously (27). Consistent with the observed inhibition of apoptotic potassium currents, TAT-DP-2, but not TAT-Sc-2, significantly attenuated TBOA-induced toxicity when compared to the scrambled peptide, measured as relative toxicity ratios ([LDH]TBOA/[LDH]Veh) (Fig. 4D). On the basis of these findings, we next evaluated the potential neuroprotective actions of TAT-DP-2 in an in vivo model of ischemia-reperfusion injury, where we have previously observed a substantial contribution of Kv2.1-enabled neuronal cell death (9).

**TAT-DP-2 induces rapid dispersal of Kv2.1 surface clusters in vivo and is neuroprotective following cerebral ischemia-reperfusion injury in mice**

Previous studies within our laboratory have validated effective delivery of small TAT-linked peptides to the cerebral vasculature and brain parenchyma within several minutes following intraperitoneal injection (9). To determine whether TAT-DP-2 could decluster Kv2.1 channels in vivo, we injected naïve young adult male mice with an intraperitoneal bolus of either TAT-DP-2 or TAT-Sc-2 (6 nmol/g). Two hours following injection, brain tissue was harvested for immunochemical staining of endogenous Kv2.1 channel distribution. We found that Kv2.1 cluster density in layers 2/3 of the cerebral cortex was significantly diminished in animals injected with TAT-DP-2, when compared with TAT-Sc-2–injected animals (Fig. 5, A to D), indicating effective TAT-DP-2–mediated Kv2.1 declustering in vivo.

We next investigated the neuroprotective efficacy of TAT-DP-2 in vivo. We used a transient, unilateral middle cerebral artery occlusion (MCAo) model of ischemic stroke in mice, which produces a highly reproducible infarct lesion involving both the striatum and cortex ipsilateral to the occlusion (28), with a significant Kv2.1-dependent cell death component (9). The experimental design for these studies (Fig. 6A) includes subjecting animals to 50 min of MCAo, followed by reperfusion. TAT-DP-2 was administered at 1 and 6 hours following the initiation of reperfusion (2 hours after vessel occlusion), as this temporal setup may recapitulate a realistic and translatable therapeutic treatment window for stroke patients undergoing thrombolytic or endovascular procedures (29). As proapoptotic Kv2.1 current enhancement likely occurs 3 to 5 hours following ischemic injury (3, 8), our goal was also to capture this critical event, ensuring that Kv2.1 clusters acting as insertion platforms for new channels reaching the membrane were adequately disrupted.

Twenty-four hours following cerebral reperfusion after MCAo, we analyzed total infarct ratios (Fig. 6B) in tetrazolium chloride–stained brain sections (Fig. 6, B and C). We found that TAT-DP-2–treated mice exhibited a robust reduction in total cerebral infarct ratio compared to TAT-Sc-2–treated animals, consistent with effective TAT-DP-2–mediated neuroprotection previously observed in vitro (Fig. 6D). Preparation of peptides, MCAo surgery, and injection, as well as infarct analysis, were all randomized and performed blindly.
Fig. 3. TAT-DP-2 displaces Kv2.1-VAPA association in cortical neurons. (A) Depiction of PLA is shown, indicating that PLA probes detect association between two target proteins of interest within 40 nm of each other. (B) Raw confocal images (60×) of immunolabeled cortical neurons are displayed, following 2-hour treatment with either TAT-Sc-2 or TAT-DP-2. PLA puncta-TRITC (tetramethyl rhodamine isothiocyanate) fluorescence indicates areas of Kv2.1-VAPA interaction. Note robust reduction in density of PLA puncta following TAT-DP-2 treatment. Scale bar, 10 µm. (C) Histograms represent PLA puncta per square micron of neuronal soma (# PLA puncta/sq. micron of cell soma) across four separate experiments. Data are displayed as means +/- SEM [Veh, 0.13 +/- 0.01 (n = 22 neurons); TAT-Sc-2, 0.10 +/- 0.06 (n = 15 neurons); TAT-DP-2 0.06 +/- 0.01 (n = 30 neurons)]. Analyzed via Kruskal-Wallis with Dunn’s MCT [Veh versus TAT-Sc-2, ns (P = 0.48); Veh versus TAT-DP-2 (**P < 0.0001); TAT-Sc-2 versus TAT-DP-2 (*P = 0.019)]. Ab, antibody; DAPI, 4',6-diamidino-2-phenylindole.
A second cohort of mice was subjected to 50-min MCAo, followed by TAT-DP-2 or TAT-Sc-2 injection at 1 and 6 hours after reperfusion (Fig. 6A). A different blinded observer then analyzed each animal and assigned an objective murine neurological score (MNS; table S1) to each mouse over a 42-day period. Because the MNS is based solely on characteristic motor deficits developed following left MCAo, all animals initially scored zero on the scale. We found that TAT-DP-2 treatment provided significant preservation of favorable neurological score when compared to scrambled control (Fig. 6E). Hence, we report a robust neuroprotective effect of TAT-DP-2 in vivo that not only reduces cerebral infarct lesion size but also preserves long-term neurological function following cerebrovascular injury in a preclinical model of stroke.

DISCUSSION

According to the World Health Organization, approximately 15 million people suffer from a stroke worldwide annually, with more than 5 million dying and another 5 million suffering from permanent, serious disability. Current therapies for ischemic stroke are limited to rapid thrombolysis or endovascular thrombus removal to restore cerebral perfusion and prevent further brain infarction. This approach is a life-saving clinical procedure, and positive outcomes have been reported with reperfusion delays of up to an astonishing 24 hours (30). This so-called reperfusion era in stroke management has called for a reevaluation of the optimal use of putative neuroprotective agents (31), especially since restoration of blood flow following the infarction could contribute to reperfusion injury, exposing patients to oxidative stress capable of inducing secondary damage. Hence, there is a critical need for the development of novel approaches to delayed neuroprotection that will supplement expedited cerebral reperfusion as a mainstay of therapy (31). Here, we targeted a key regulator of neuronal apoptosis within the ischemic penumbra: the Kv2.1 potassium channel. As Kv2.1-enabled cell death is likely delayed following the onset of injury (3, 8, 25) and occurs by a well-defined mechanism (fig. S7, A and B), it offers an attractive therapeutic target for neuroprotection during the reperfusion and postreperfusion period.

Following oxidative or nitrosative injury, free zinc displacement from metallothionein by reactive oxygen intermediate species (ROS) triggers dual phosphorylation of Kv2.1 channels by Src and p38, resulting in a calcium/calmodulin-dependent protein kinase II–dependent interaction between syntaxin and the proximal CT domain of the channel (3, 8, 10, 25, 32–34). This process results in exocytotic insertion of proapoptotic Kv2.1 channels into the PM, likely at specialized Kv2.1 cluster domains that form ER-PM junctions.
Fig. 5. Intraperitoneal injection of TAT-linked DP-2 in mice induces rapid dispersal of Kv2.1 surface clusters. (A) Confocal images (60x) of 30-μm coronal mouse brain sections of superficial cortex are shown, following intraperitoneal (IP) injection of live mice with either TAT-Sc-2 (6 nmol/g; n = 3 animals) or TAT-DP-2 (6 nmol/g; n = 3 animals), sacrificed 2 hours after injection. Fluorescein isothiocyanate (FITC) fluorescence indicates immunolabeled endogenous Kv2.1 channels. Note preservation of highly localized Kv2.1 surface clusters in brain tissue from animals injected with TAT-DP-2 and robust dispersal of Kv2.1 surface clusters following TAT-DP-2 injection. Scale bar, 10 μm. (B) Identical paired images to those in (A) are displayed, highlighting automated Kv2.1 cluster analysis. Areas of green intensity indicate cluster domains. (C) Zoomed images of single neurons from each treatment group are displayed, correlating with the white boxes from (A). Scale bar, 10 μm. (D) Histograms represent mean number Kv2.1 cluster density (number of Kv2.1 clusters per image field) across three separate experiments, using three separate mice per treatment group. Six separate brain slices were obtained and imaged from each mouse to yield 51 to 54 image fields per treatment group. Data are displayed as means ± SEM [TAT-Sc-2, 565.70 ± 50.72 (n = 51 fields); TAT-DP-2, 301.10 ± 39.60 (n = 54 fields)]. Analyzed via Mann-Whitney test (****P < 0.0001).
Fig. 6. Intraperitoneal injection of mice with TAT-DP-2 reduces cerebral infarct volume. (A) Time points for MCAo and peptide injection. (B) Infarct analysis method. (C) Top: Paired coronal brain sections from mice intraperitoneally injected with either TAT-Sc-2 or TAT-DP-2 following 50-min left MCAo, peptide injection, and 2,3,5-triphenyltetrazolium chloride (TTC) staining. Bottom: Single sections (slice 2) are displayed from different mice following the same protocol. Photo credit: Anthony J. Schulien, University of Pittsburgh School of Medicine. (D) Histograms represent mean total infarct ratio of animals following 50-min MCAo and intraperitoneal injection with either TAT-Sc-2 or TAT-DP-2. Data are displayed as means ± SEM [TAT-Sc-2, 0.121 ± 0.009 (n = 9 animals); TAT-DP-2, 0.071 ± 0.020 (n = 8 animals)]. Analyzed via unpaired t test (*P = 0.0335). (E) Plots of MNS versus time are displayed for cohorts intraperitoneally injected with either TAT-Sc-2 (blue; n = 5 mice) or TAT-DP-2 (green; n = 8 mice) following 50-min left MCAo. MNS indicates blinded assessment of neurological function, where 0 is no neurological deficit and 8 is stroke-related death (table S1). Analyzed via two-way ANOVA for peptide treatment effect between groups (***P = 0.0007).
noncanonical FFAT motifs found on both Kv2.1 and Kv2.2 also mutated into its pseudo-phosphorylated form. Furthermore, isolated tide spot array assay argues that the active peptide fragment from treatment reduces the interaction between Kv2.1 and VAPA, a peptide based on the critical region of Kv2.2 CT that induces Kv2.1 cluster dispersal, we demonstrate Kv2.1 cluster dispersal and neuroprotection in vitro and in vivo. We observe improved neurologic response, physiologic Kv2.1 cluster dispersal is often transient and facilitates insulin exocytosis (35, 36), following stroke in mice, the area of these junctions increases significantly, accompanied by endogenous Kv2.1 channel cluster dispersal. Inhibition of the P2Y12 receptor, thought to mediate these microglial somatic contacts, following stroke leads to an increase in infarct damage. Thus, this study further implicates the Kv2.1 cluster domain as a critical player in the neuronal response to ischemic insult.

Previous preclinical studies from our laboratory have shown efficacious neuroprotection in vivo by targeting the Kv2.1-syntaxin interaction (fig. S7B), which is the final step in proapoptotic Kv2.1 membrane insertion (9, 11). The results in the present study target this process via a completely novel approach, namely, the elimination of the putative insertion sites for cell death trafficking of channels, the Kv2.1 membrane cluster domains. Given that this study reports a second separate mechanism of effective Kv2.1-specific targeted neuroprotection following ischemic injury, it is likely that further translational development of these strategies, or even their synergistic use, will lead to the development of a novel class of delayed, penumbral neuroprotectant drugs. Furthermore, as a multitude of neurodegenerative disorders may rely on apoptotic cell death for progression (38, 39), this strategy targets a highly conserved mechanism with a novel approach that may even provide a generalizable class of antiapoptotic drugs for disorders including Alzheimer’s disease and Parkinson’s disease.

Although the PLAs in our study clearly show that TAT-DP-2 treatment reduces the interaction between Kv2.1 and VAPA, a peptide spot array assay argues that the active peptide fragment from DP-2 (SIDSFTS) does not bind to VAPA proteins directly, even when mutated into its pseudo-phosphorylated form. Furthermore, isolated noncanonical FFAT motifs found on both Kv2.1 and Kv2.2 also yielded no binding to VAPA proteins in peptide spot array studies, although recent work suggests that this interaction may be critical for Kv2.1 cluster formation. Mutation of the FFAT binding domain on VAPA abolishes the ability for Kv2.1 channels to form clusters in human embryonic kidney (HEK) cells in vitro (17, 18). One possible explanation for this inconsistency is simply that peptide spot array studies do not accurately allow for tertiary or quaternary protein structure formation that is required for interaction and binding. Furthermore, a larger fragment of the CT domain of Kv2.1 or Kv2.2 may be required for total VAPA binding, and TAT-DP-2 might simply be outcompeting existing Kv2.1 channels by blocking a smaller portion of the FFAT binding domain located on VAPA proteins. It is also plausible that the actions of TAT-DP-2 require Kv2.1-VAPA association in the cellular cluster microdomain, while acellular experiments such as the peptide spot array do not provide a sufficiently realistic spatial and electrostatic articulation between key proteins. Another possibility is that TAT-DP-2 may have a target sequence within Kv2.1 itself, and that, upon binding, does not allow the channel to bind VAPA via an allosteric mechanism. Understanding this biophysical association may prove invaluable for further development of additional neuroprotective peptides and small molecules that displace this interaction, as well as for optimization of our current strategy. However, the lack of Kv2.1 cluster dispersal following treatment with a TAT-linked canonical FFAT motif (fig. 55, B and C) suggests that DP-2’s declustering-inducing disruption of Kv2.1-VAPA association might occur via a unique mechanism and thus could lack significant off-target actions on the broad array of VAP-binding proteins.

Last, the steps required to translate these findings to clinical practice have a strong existing precedent, as TAT-linked neuroprotective peptides targeting NMDA receptor-mediated production of nitric oxide have shown efficacy in phase 2 and 3 clinical trials (29, 40). However, the optimal timing of TAT-DP-2 administration in a clinical stroke scenario merits further study. With new data that enable the use of endovascular thrombectomy and reperfusion at later time points following stroke (30, 41), the clinical utility of neuroprotective agents may be increasing significantly. While TAT-DP-2 provides efficacious ischemic neuroprotection when administered following reperfusion, it may be important to evaluate its effects when administered before reperfusion (42). Aside from impacts on the brain, one unique consideration that will need to be studied in the process of translating these findings to clinical practice is the impact of Kv2.1 channel declustering on pancreatic β cell function. As studies have shown that Kv2.1, but not Kv2.2, forms clusters on pancreatic β cells and facilitates insulin exocytosis (43), it is possible that rapid Kv2.1 declustering could alter blood insulin levels, albeit very transiently.

In summary, we show that targeted dispersal of Kv2.1 channel clusters both reduces infarct area and preserves neurofunctional function following ischemic stroke in mice. Furthermore, we define the mechanism of this neuroprotective effect and provide proof of concept for an injectable neuroprotective therapeutic. Using a TAT-linked peptide based on the critical region of Kv2.2 CT that induces Kv2.1 cluster dispersal, we demonstrate Kv2.1 cluster dispersal and neuroprotection in vitro and in vivo. We observe improved neurologic function in peptide-treated animals for up to 6 weeks following an induced cerebral infarct. Our data also present strong mechanistic evidence that disruption of Kv2.1-VAPA interaction by our peptide can account for the neuroprotective blockade of proapoptotic Kv2.1 potassium currents following injury (fig. S7). Our results strongly cement the notion that targeting Kv2.1-facilitated cell death is likely
to yield effective, innovative, neuroprotective therapeutic drugs for patients suffering from ischemic stroke and, in future studies, the many other neurodegenerative disorders where this channel has been implicated (44–46).

**MATERIALS AND METHODS**

**Study design**

The objective of the study was to define the critical region within the Kv2.2 CT that is responsible for Kv2.1 declustering and to develop a neuroprotective declustering therapeutic based on this sequence. For in vitro experiments using neuronal cell culture for confocal imaging or toxicity studies, experiments were reproduced at least three times on three separate culture dates. For in vivo MCAo experiments, results of a small pilot study were used to power sample sizes.

**Ethical approval**

The Institutional Animal Care and Use Committee at the University of Pittsburgh School of Medicine approved all animal protocols detailed in this study.

**Neuronal cell culture**

In vitro experiments used primary cortical neuronal cultures prepared from embryonic day 16 to 17 Sprague-Dawley rats of either sex in a protocol described previously (10). Timed-pregnant rats (Charles River Laboratories, Wilmington, MA) were sacrificed by CO₂ inhalation. Embryonic cortices were dissociated with trypsin (0.6 mg/ml), and cells were plated in sterile six-well plates on poly-1-ornithine-coated glass coverslips at a density of 670,000 cells per well. Cytosine arabinoside (1 to 2 μM) was used to inhibit nonneuronal proliferation on day 14 in vitro (DIV); cultures were used at 21 to 25 DIV.

**TAT-linked peptides**

The UniProt Consortium protein sequence database was used to align protein sequences. TAT (YGRKKRRQRRR)–linked peptides with 95.1 to 99.8% purity analyzed by high-performance liquid chromatography were generated by GenScript (Piscataway, NJ) (table S2). Notably, clustering residues within the PRC domain of Kv2.1 (S587, S590, F591, and S593; rat sequence, UniProt P15387) correspond to S83, S86, F87, and S89 in (20).

**Plasmid constructs**

pCMV-DP-1 and pCMV-Sc-1 plasmids were generated by standard cloning into the multiple cloning sites of pCMV-IRE52-GFP (Clontech, catalog no. 6029-1). The IRES2-GFP region of the parent plasmid was subsequently removed. Successful cloning was confirmed by sequencing. Table S3 lists all plasmids used in this study.

**Transfection**

Transfections were performed with Lipofectamine 2000 (L2K; Invitrogen, Carlsbad, CA). Cortical neuronal cultures on glass coverslips were placed into wells containing 500 μl of Dulbecco’s minimum essential medium with 2% HyClone bovine serum (D2C, Thermo Fisher Scientific, Waltham, MA). A mixture of 1.5 μg of total cDNA and 2 μl of L2K was prepared in 100 μl of Opti-MEM I (Life Technologies Corp., Grand Island, NY) for each well and added. Cultures were used 18 to 24 hours following transfection. cDNA amounts for each transfection experiment are described below for each figure [Fig. 1: Kv2.1-GFP plasmid (0.375 μg per well), pcDNA3 vector plasmid (0.495 μg per well), and either DP-1 or Sc-1 or Kv2.2 CT–expressing plasmid (0.63 μg per well); Fig. 2 and figs. S2 to S4 and S6: Kv2.1-GFP plasmid (0.375 μg per well), tdTomato plasmid (0.15 μg per well) (from G. Ryffel; Addgene plasmid no. 30530), and pcDNA3 vector plasmid (0.975 μg per well)].

**Immunohistochemistry**

Eight-week-old C57BL/6j males (28 to 30 g; The Jackson Laboratory, Bar Harbor, ME) were administered intraperitoneal injections of TAT-DP-2 (6 nmol/g; n = 3) or TAT-Sc-2 peptide (6 nmol/g; n = 3). Two hours after injection, mice were anesthetized with 3% isoflurane (Henry Schein Animal Health, Dublin, OH) in a 3:1 NO/O₂ gas mixture. Fifty milliliters of ice-cold 1x sterile phosphate-buffered saline (PBS) was transcardially perfused (5 to 10 ml/min), immediately followed by 50 ml of 4% sterile paraformaldehyde (PFA; 5 to 10 ml/min). Brains were removed and placed in 4% PFA at 4°C for 24 hours and then transferred to 30% sucrose in 1× PBS at 4°C for 48 to 72 hours. Brains were embedded with Tissue-Tek OCT Compound (Sakura Finetek USA Inc., Torrance, CA) onto an SM2010R cryosectioner (Leica Biosystems, Wetzlar, Germany), sectioned (30 μm), and stored.

Brain sections were rinsed in sterile 24-well plates in tris-buffered saline (TBS) solution. Sections were then transferred to new wells containing 0.5% Triton X-100 and 10% normal goat serum (NGS) in TBS for 30 min at room temperature. Next, sections were again transferred to wells containing primary anti-Kv2.1 antibody [mouse monoclonal immunoglobulin G (IgG), RRID AB_192761, University of California (UC) Davis/NeuroMab Facility], diluted 1:200 in 0.3% Triton X-100 and 3% NGS in TBS for 24 hours at 4°C. Sections were washed in TBS and incubated with secondary anti-mouse IgG Sigma fluorescein isothiocyanate–tagged antibody (goat monoclonal IgG; catalog F0257, Sigma-Aldrich, St. Louis, MO), diluted 1:500 in 0.3% Triton X-100 and 3% NGS in TBS for 1 hour at room temperature, and then mounted.

**Immunocytochemistry**

The in vitro Kv2.1 immunofluorescence experiment (fig. S4) was carried out in DIV 21 to 26 primary rat neuronal cultures, as described above. Each coverslip was treated with either TAT-DP-2 or TAT-Sc-2 in its individual well to reach a final concentration of 10 μM. After 3 hours, coverslips were transferred to PBS and immediately fixed with 4% PFA/4% sucrose for 15 min. Coverslips were rinsed with PBS three times and then permeabilized with 0.25% Triton X-100 for 15 min. Cells were rinsed with PBS three times and then blocked with 2% bovine serum albumin (BSA)/10% NGS for 1 hour at room temperature. Neurons were stained with mouse anti-Kv2.1 antibody (1:250; clone K89/34, Abcam, RRID: AB_2750677) using the microdrop method at 4°C overnight. Cells were rinsed in PBS three times and then stained with Alexa Fluor 568 goat anti-mouse secondary antibody (1:500; Thermo Fisher Scientific, RRID: AB_2534072) for 1 hour at room temperature. Last, cells were rinsed in PBS three times before mounting. Confocal Z projections of well-isolated neurons were obtained for evaluation of Kv2.1 cluster density (described below). The experimenter was blinded to the treatment groups during image analysis.

**Confocal imaging and Kv2.1 cluster analysis**

To analyze Kv2.1 distribution in live primary cortical neurons, cells were transfected with both a tdTomato construct and a GFP-tagged Kv2.1 construct (21) and imaged on a Nikon A1+ confocal microscope.
Schulien et al., Sci. Adv. 2020; 6 : eaaz8110     1 July 2020

Primary cortical neurons on glass coverslips were treated in 24-well plates with vehicle (dH2O), TAT-Sc-2 (10 μM), or TAT-DP-2 (10 μM) for 2 hours at 37°C/5% CO2. Neurons were then rinsed with 2 ml of ice-cold sterile PBS and then transferred to 1 ml of 4% PFA in PBS for 20 min for fixation. Coverslips were transferred to 1 ml of 0.1 M glycine (in PBS) to quench fixation and rinsed three times in 1 ml of PBS. Membrane permeabilization was accomplished with 1 ml of 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and nonspecific binding was blocked with 10% BSA (in PBS) for 30 min. Primary immunolabeling was accomplished by 18 to 24 hours of exposure to rabbit anti-Kv2.1 polyclonal antibody (1:500 in 3% BSA; RRID AB_2040162, Alomone Labs, Jerusalem, Israel), mouse anti-VAPA monoclonal antibody (1:500 in 3% BSA; RRID AB_2722702, UC Davis/NeuroMab Facility), and chicken anti-MAP2 polyclonal antibody (1:1000 in 3% BSA; RRID AB_5392, Abcam, Cambridge, MA) in 3% BSA (in PBS) at 4°C under gentle agitation. Secondary immunolabeling of MAP2 staining was accomplished by incubation with a Cy5-tagged donkey anti-chicken antibody (1:500 in 3% BSA) for 1 hour. Coverslips were then rinsed four times for 5 min in PBS before beginning the PLA. Duolink protocol was followed according to factory instructions with adaptation from (24). Briefly, fixed and immunocytochemically labeled neurons were first incubated with oligonucleotide-conjugated anti-mouse minus (1:5 in 3% BSA) and anti-rabbit plus (1:5 in 3% BSA) probes for 1 hour at 37°C to probe for anti-VAPA and anti-Kv2.1 primary antibodies, respectively. Coverslips were then rinsed in Duolink PLA Wash Buffer A two times for 5 min. Oligonucleotide ligation of plus and minus probes was accomplished by incubation with ligase (1:40) in Duolink PLA ligation solution (1:5 in dH2O) for 45 min at 37°C, before coverslips were again rinsed in Wash Buffer A two times for 2 min. Rolling circle amplification reaction and subsequent fluorescently labeled oligonucleotide hybridization with this concatemeric product was accomplished by incubating coverslips with Duolink amplification orange (1:5 in dH2O) and polymerase enzyme (1:40) for 100 min at 37°C. Last, coverslips were washed in Duolink Wash Buffer B two times for 10 min, followed by a final wash in 0.01× Wash Buffer B for 1 min. Coverslips were then mounted onto glass slides with Duolink 4′,6-diamidino-2-phenylindole mounting media for confocal imaging.

PLA puncta were imaged on a Nikon A1+ confocal microscope (Nikon, Tokyo, Japan) with a 60× objective. Random neuronal somas were identified on each coverslip using the Cy5 filter to visualize MAP2 staining. PLA puncta signals were then obtained by capturing 25 optical sections (0.5 μm) that constrained the entire Z width of PLA-TRITC (tetrathymethyl rhodamine isothiocyanate) fluorescence as MAX-IP files. Laser power, HV (gain), offset, pinhole size, and all camera parameters were kept identical between all samples in four separate experiments. NIS-Elements AR object count feature was then used to analyze the quantity of PLA puncta per cell. To do this, cell somas and proximal dendrites were first selected as ROIs, using MAP2-positive staining to confirm analysis of neurons. As distinct local PLA puncta-TRITC autofluorescence (markedly different in appearance from PLA puncta) was noted, local background subtraction using a small ROI within this region was performed in a protocol similar to that described earlier (48). Next, PLA puncta were counted and measured by a semi-automated object count feature within the NIS-Elements AR analysis program. Thresholding was set for each image by choosing well-defined, highly fluorescent PLA puncta compared to background fluorescence and setting inclusion thresholds based on these puncta. Object counts were verified by manual counting.

Electrophysiology

Whole-cell voltage clamp currents from rat cortical neurons were obtained with Axopatch 200b amplifier using pClamp software (Molecular Devices, Sunnyvale, CA) using 3- to 5-megohm electrodes. The extracellular solution contained the following: 2.0 mM MgCl2, 2.5 mM KCl, 115 mM NaCl, 10 mM Hepes, 10 mM p-glucose, 1.0 mM CaCl2, and 0.25 μM tetrodotoxin (pH 7.2). The electrode solution...
contained the following: 100 mM potassium gluconate, 1 mM MgCl₂, 10 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂–adenosine triphosphate, 0.33 mM guanosine triphosphate, 11 mM EGTA, and 10 mM Hepes (pH 7.2). Series resistance was compensated (80%), and currents were digitized at 10 kHz and filtered at 2 kHz. Potassium currents were evoked with a series of 200-ms voltage steps from a holding potential of −80 to 80 mV in 10-mV increments. A 30-ms pre-pulse to +10 mV was used to inactivate A-type potassium currents. Delayed rectifier currents were measured relative to baseline at 180 ms after the initiation of each voltage step. Currents were normalized to cell capacitance. Current density analyses were limited to the +30-mV step to minimize voltage errors due to large whole-cell current amplitudes at higher holding potentials, which can be significant even after compensation for series resistance.

Cell toxicity assay
Cortical neurons were pretreated with vehicle, TAT- DP-2 (3 μM), or TAT-Sc-2 (3 μM) for 2 hours in Hepes-buffered minimal essential media with 0.01% BSA (MHB). Next, cells were incubated with the same peptide concentrations along with either vehicle or 60 μM TBOA for 6 hours. Each well was washed twice with either 0.5 ml of vehicle or 60 μM TBOA, and neurons were incubated overnight. Twenty-four hours following initial TBOA treatment, medium was collected for LDH assays (Sigma-Aldrich, St. Louis, MO). Relative toxicity was quantified by calculating the ratio of LDH concentration in TBOA-treated over non–TBOA-treated wells. In cell culture, LDH release measures neuronal cell death and is not specific to any one mechanism of cell demise.

MCAo microsurgery
Mice were anesthetized with isoflurane, and the common carotid artery was exposed. A single-use silicon-coated suture (MCAo suture no. 602256PK10, Doccol Corporation, Sharon, MA) was advanced 8 to 9 mm into the internal carotid artery, occluding the MCA. The suture was secured with 6-0 silk ligatures for 50 min and then removed. Twenty-four hours following cerebral reperfusion, mice were sacrificed, and four 2-mm coronal brain sections were obtained from each animal. Sections were stained with 0.05% 2,3,5-triphenyltetrazolium chloride in PBS for 30 min at room temperature. Brain sections were digitally scanned following staining, and a blinded experimenter measured infarct ratios (infarct area/total section area) using National Institutes of Health (NIH) ImageJ software. Visual representation of this protocol is in Fig. 6B.

To analyze neurological deficits following MCA stroke, testing was performed by a blinded experimenter on additional cohorts, both at baseline (all initial scores were 0) and on post-stroke days 1, 2, 3, 5, 7, 10, 14, 21, 28, 35, and 42. An objective MNS (table S1) was assigned on each day, ranging from 0 (no neurological deficit) to 8 (stroke-related death) (9).

Peptide-blot array
Standard 9-fluorenylmethoxycarbonyl carbonyl chemistry was used to synthesize peptides and spot them onto CelluSpots nitrocellulose disks prederivatized with a polyethylene glycol spacer (Intavis), using the ResPep peptide synthesizer. The peptides were spotted on 20 membranes fitted on microscope glass slides (Intavis) using an Intavis MultiPep robot. For hybridization and immunoblotting of arrays, CelluSpots slides were washed in TBST [50 mM tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20] for 10 min and 5% nonfat dry milk and then blocked for 1 hour at room temperature with gentle shaking in TBST containing 5% nonfat dry milk. Lysates from HEK293 cells transfected as before (9, 11, 49) with pEGFP-N1–VAPA, 1-242 (Addgene no. 18874) were made in 20 mM tris, 50 mM NaCl, 2 mM MgCl₂, 1% Terrigel, 0.5% sodium deoxycholate, 0.1% SDS with protease (catalog no. B14402, Bimake), phosphatase (catalog no. B15002, Bimake) inhibitor cocktails, and universal nuclease (5 U/ml; catalog no. 88702, Thermo Fisher Scientific) added extemporaneously. The concentration of lysates was measured by bicinchoninic acid (catalog no. 23227, Thermo Fisher Scientific), and peptide arrays were incubated with total protein (1 mg/ml) overnight at 4°C (9, 11, 50). Peptide arrays were washed three times for 5 min at room temperature with TBST and incubated with the primary antibody anti-VAPA (RRID AB_2722707, UC Davis/NIH NeuroMab Facility) for 2 hours at room temperature with gentle shaking in TBST and 5% BSA. After washing with TBST, the membranes were incubated in secondary antibody (IRDye 800CW Goat anti-Mouse IgG secondary antibody; RRID AB_621842, LI-COR) for 45 min, washed three times for 5 min in TBST, and visualized by infrared fluorescence (LI-COR). Four independent peptide spot arrays were used in this study. Fluorescence intensity was analyzed with ImageJ software (LI-COR).

Statistical analyses
Data are presented as means ± SEM. All statistical analyses were performed in GraphPad Prism 7 (GraphPad, San Diego, CA) or BioVinci data analysis software. For comparison of two sample means, an unpaired two-tailed t test was used for parametric data; Mann-Whitney tests were used for nonparametric data. For comparison of more than two sample means, a one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test (MCT) for relevant comparisons was used; Kruskal-Wallis tests with Dunn’s MCT were used for nonparametric data. For behavioral studies, a two-way ANOVA was used to compare a peptide treatment effect between the two sample cohorts.

Supplementary materials
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/27/eaaz8110/DC1

View/request a protocol for this paper from Bio-protocol.

References and notes
1. M. G. George, L. Fischer, W. Koroshetz, C. Bushnell, M. Frankel, J. Foltz, P. G. Thorpe, CDC grand rounds: Public health strategies to prevent and treat strokes. MMWR Morb. Mortal. Wkly Rep. 66, 479–481 (2017).
2. B. R. S. Broughton, D. C. Reutens, C. G. Sobey, Apoptotic mechanisms after cerebral ischemia. Stroke 40, e331–e339 (2009).
3. S. Pal, K. A. Hartnett, J. M. Nerbonne, E. S. Levitan, E. Aizenman, Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. J. Neurosci. 23, 4798–4802 (2003).
4. H. Murakoshi, J. S. Trimmer, Identification of the Kv2.1K⁺ channel as a major component of the delayed rectifier K⁺ current in rat hippocampal neurons. J. Neurosci. 19, 1728–1735 (1999).
5. D. Guan, W. E. Armstrong, R. C. Foehring, Kv2 channels regulate firing rate in pyramidal neurons from rat sensorimotor cortex. J. Physiol. 591, 4807–4825 (2013).
6. F. M. Hughes Jr., C. D. Bortner, G. D. Purdy, J. A. Cidlowski, Intracellular K⁺ suppresses the activation of apoptosis in lymphocytes. J. Biol. Chem. 272, 30567–30576 (1997).
7. S. P. Yu, C.-H. Yeh, S. L. Sensi, B. J. Gwag, L. M. T. Canzoniero, Z. S. Farhangrazi, H. S. Ying, M. Tian, L. L. Dugan, D. W. Choi, Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 278, 114–117 (1997).
8. S. K. Pal, K. Takimoto, E. Aizenman, E. S. Levitan, Apoptotic surface delivery of K⁺ channels. Cell Death Differ. 13, 661–667 (2006).
9. C.-Y. Yeh, A. M. Bulas, A. Moutal, J. L. Saloman, K. A. Hartnett, C. T. Anderson, T. Tzounopoulos, D. Sun, R. Khanna, E. Aizenman, Targeting a potassium channel/syntaxin interaction ameliorates cell death in ischemic stroke. J. Neurosci. 37, 5648–5658 (2017).
16. S. Baver, K. M. S. O'Connell, The C-terminus of neuronal Kv2.1 channels is required for channel localization and targeting but not for NMDA-receptor-mediated regulation of channel function. *Neuroscience* **354**, 158–167 (2017).

17. M. Kirmiz, N. C. Vierra, S. Palacio, J. S. Trimmer, Identification of VAPA and VAPB as Kv2.1 cell surface clusters is insertion platforms for ion channel delivery to the plasma membrane. *Mol. Biol. Cell* **23**, 2917–2929 (2012).

18. B. Johnson, A. N. Leek, L. Solé, E. E. Maverick, T. P. Levine, M. M. Tamkun, Kv2 potassium channels form endoplasmic reticulum/plasma membrane junctions via interaction with VAPA and VAPB. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E7331–E7340 (2018).

19. B. Johnson, A. N. Leek, M. M. Tamkun, Kv2 channels create endoplasmic reticulum/plasma membrane junctions: A brief history of Kv2 channel subcellular localization. *Channels* (Austin) **13**, 88–101 (2019).

20. S. T. Lim, D. E. Antonucci, R. H. Scannevin, J. S. Trimmer, A novel targeting signal for proximal clustering of the Kv2.1 K⁺ channel in hippocampal neurons. *Neuron* **25**, 385–397 (2000).

21. M. K. S. O’Connell, A. S. Rolig, J. D. Whitesell, M. M. Tamkun, Kv2.1 potassium channels are retained within dynamic cell surface microdomains that are defined by a perimeter fence. *J. Neurosci.* **26**, 9069–9018 (2006).

22. S. R. Schwarzau, A. Ho, A. Vucero-Akbari, S. F. Dowdy, In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science* **285**, 1569–1572 (1999).

23. J. M. Britain, D. B. Duarte, S. M. Wilson, W. Zhu, C. Ballard, P. L. Johnson, I. Katona, Identification of VAPA and VAPB as Kv2 channel-interacting proteins defining endoplasmic reticulum–plasma membrane junctions in mammalian brain neurons. *J. Neurosci.* **30**, 7562–7584 (2010).

24. B. Johnson, A. N. Leek, S. I. Savitz, J.-C. Baron, M. A. Yenari, N. Sanossian, M. Fisher, Reconsidering neuroprotection in the reperfusion era. *Stroke* **48**, 3413–3419 (2017).

25. M. McCord, E. Aizenman, Convergent Ca²⁺ and Zn²⁺ signaling regulates apoptotic Kv2.1 K⁺ currents. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 13988–13993 (2013).

26. P. T. Redman, K. A. Hartnett, M. A. Aras, E. S. Leviatan, E. Aizenman, Regulation of apoptotic potassium currents by coordinated zinc-dependent signalling. *J. Physiol.* **587**, 4393–4404 (2009).

27. P. T. Redman, K. He, K. A. Hartnett, B. S. Jefferson, L. Hu, P. A. Rosenberg, E. S. Leviatan, E. Aizenman, Apoptotic surge of potassium currents is mediated by p38 phosphorylation of Kv2.1. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3568–3573 (2007).

28. H. Misonou, S. M. Thompson, X. Cai, Dynamic regulation of the Kv2.1 voltage-gated potassium channel during brain ischemia through neuroglial interaction. *J. Neurosci.* **28**, 8529–8538 (2008).

29. H. Misonou, D. P. Mohapatra, E. W. Park, V. Leung, D. Zhen, K. Misonou, A. E. Anderson, J. S. Trimmer, Regulation of ion channel localization and phosphorylation by neuronal activity. *Nat. Neurosci.* **7**, 711–718 (2004).

30. C. Cserép, B. Pósfai, N. Lénárt, R. Fekete, Z. I. László, Z. Lele, B. Orsiolts, G. Molnár, S. Heindl, A. D. Schwarzh, K. Ujvári, Z. Környei, K. Tóth, E. Szabadsitz, B. Sperlagh, M. Baranyi, L. Csiba, T. Hortobágyi, Z. Maglóczky, B. Martinez, G. Szabó, F. Erdélyi, R. Szépics, M. M. Tamkun, B. Gesierich, M. Duering, I. Katona, A. Liesz, G. Tamás, A. Dénes, Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. *Science* **367**, 528–537 (2020).

31. R. M. Friedlander, Apoptosis and caspases in neurodegenerative diseases. *N. Engl. J. Med.* **318**, 1365–1373 (2005).

32. M. P. Mattson, Apoptosis in neurodegenerative disorders. *Nat. Rev. Mol. Cell Biol.* **1**, 120–129 (2000).

33. M. D. Hill, R. H. Martin, P. D. Mikulis, J. H. Wong, F. I. Silver, K. G. terBrugge, P. G. Milot, W. M. Clark, R. Loch MacDonald, M. E. Kelly, M. Boulton, I. Fleetwood, C. M. Dougall, T. Gunnarsson, M. Chow, C. Lum, R. Dodd, J. Poubil, P. T. Kings, A. M. Demchuk, P. M. Goyal, R. Anderson, J. Bishop, D. Garman, P. M. Timans; ENACT trial investigators, Safety and efficacy of NA-1 in patients with iatrogenic stroke after endovascular aneurysm repair (ENACT): A phase 2 randomised, double-blind, placebo-controlled trial. *Lancet Neurol.* **11**, 942–950 (2012).

34. G. W. Albers, M. P. Marks, S. Kemp, S. Christensen, J. P. Tsai, S. Ortega-Gutierrez, R. A. McTaggart, M. T. Torbay, M. K. Temsizer, T. Leslie-Mazwi, A. Sarraj, S. E. Kasner, S. A. Ansari, S. D. Yeatts, S. Hamilton, M. Mlynash, J. J. Heit, G. Zaharchuk, S. Kim, J. Carrozzella, Y. Y. Palesch, A. M. Demchuk, R. Bammer, P. W. Lavori, J. P. Broderick, G. M. Lansberg; DEFUSE Investigators, Thrombectomy for stroke at 6 to 16 hours with selection by perfusion imaging. *N. Engl. J. Med.* **378**, 708–718 (2018).

35. M. Goyal, B. K. Menon, W. H. van Zwam, D. J. W. Dippel, P. J. Mitchell, A. M. Demchuk, P. A. Davalos, C. B. I. M. Majorje, A. van de Lucht, M. A. de Miquel, G. A. Donnan, Y. B. E. M. Roos, P. A. Bonafe, R. Jahan, H.-C. Diener, L. A. van den Berg, E. I. Levy, O. A. Berkhemer, V. M. Pereira, J. Rempel, M. Millan, S. M. Davis, P. D. Roy, J. Thornton, L. S. Roman, M. Ribó, D. Beumer, P. B. Stouch, S. Brown, B. C. V. Campbell, R. J. van Oostenbrugge, J. L. Saver, M. D. Hill, T. J. G. Jovin; HERMES collaborators, Endovascular thrombectomy after large-vessel ischaemic stroke: A meta-analysis of individual patient data from five randomised trials. *Lancet* **387**, 1723–1731 (2016).

36. J. Fu, X. Dai, G. Plummer, K. Suzuki, A. Bautista, J. M. Githaka, L. Senior, M. Jensen, D. Greitzer-Antes, J. E. Palesch, A. M. Demchuk, R. A. McTaggart, M. T. Torbey, M. Kim-Tenser, T. Leslie-Mazwi, A. Sarraj, S. E. Kasner, R. J. van Oostenbrugge, J. L. Saver, M. D. Hill, T. G. Jovin; HERMES collaborators, Endovascular thrombectomy after large-vessel ischaemic stroke: A meta-analysis of individual patient data from five randomised trials. *Lancet* **387**, 1723–1731 (2016).

37. D. Greitzer-Antes, J. E. Manning Fox, H. Y. Gaisano, C. B. Newgard, N. Touret, P. E. MacDonald, Kv2.1 clustering contributes to insulin exocytosis and rescues human iPSC-derived beta-cell dysfunction. *Diabetes* **66**, 1890–1900 (2017).

38. M. C. McCord, E. Aizenman, The role of intracellular zinc release in aging, oxidative stress, and Alzheimer’s disease. *Front. Aging Neurosci.* **6**, 77 (2014).

39. Y. Wei, M. R. Shin, F. Sesti, Oxidation of KCNB1 channels in the human brain and in mouse model of Alzheimer’s disease. *Cell Death Dis.* **9**, 820 (2018).

40. W. Yu, R. Parakramaweera, S. Teng, M. Gowda, Y. Sharad, S. Thakker-Varia, F. Sesti, Oxidation of KCNB1 potassium channels causes neurotoxicity and cognitive impairment in a mouse disease model of a genetic brain injury. *J. Neurosci.* **36**, 11084–11096 (2016).

41. A. J. Schulien, J. A. Justice, R. Di Maio, Z. P. Wills, N. H. Shah, E. Aizenman, Zn²⁺–induced Ca²⁺ release via ryanodine receptors triggers calcineurin-dependent neuroprotection and excitotoxicity in a mouse model of Alzheimer’s disease. *Front. Aging Neurosci.* **10**, 281 (2018).
redistribution of cortical neuronal Kv2.1 K⁺ channels. J. Physiol. 594, 2647–2659 (2016).

48. M. W. Glynn, A. K. McAllister, Immunocytochemistry and quantification of protein colocalization in cultured neurons. Nat. Protoc. 1, 1287–1296 (2006).

49. E. T. Duhrude, A. Moutal, X. Yang, Y. Wang, M. Khanna, R. Khanna, Hierarchical CRMP2 posttranslational modifications control NaV1.7 function. Proc. Natl. Acad. Sci. U.S.A. 113, E8443–E8452 (2016).

50. A. Moutal, Y. Wang, X. Yang, Y. Ji, S. Luo, A. Dorame, S. S. Bellampalli, L. A. Chew, S. Cai, E. T. Duhrude, J. E. Keener, M. T. Marty, T. W. Vanderah, R. Khanna, Dissecting the role of the CRMP2-neurofibromin complex on pain behaviors. Pain 158, 2203–2221 (2017).

Acknowledgments: We thank B. Kühn (University of Pittsburgh School of Medicine) for valuable feedback and guidance and K. Hartnett, G. Kosobucki, and S. Guar (University of Pittsburgh School of Medicine) for valuable technical support. We thank G. Begum for technical guidance on immunohistochemical studies. We thank B. Molyneaux for valuable feedback on the clinical context of this work. Funding: This work was funded by NIH grant NS043277 to E.A. A.J.S. was supported, in part, by Physician Scientist Training Program Scholarship. Author contributions: E.A. funded the work. E.A., A.J.S., J.A.J., and D.S. designed the experiments. E.A., A.J.S., J.A.J., C.-Y.Y., B.N.O., O.J.P., M.P.H., A.M., and R.K. collected and analyzed data. A.J.S. and E.A. wrote the manuscript. All authors critically edited and approved the final manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. Contact E.A. (redox@pitt.edu) for data files or materials.

Submitted 10 October 2019
Accepted 15 May 2020
Published 1 July 2020
10.1126/sciadv.aaz8110

Citation: A. J. Schulien, C.-Y. Yeh, B. N. Orange, O. J. Pav, M. P. Hopkins, A. Moutal, R. Khanna, D. Sun, J. A. Justice, E. Aizenman, Targeted disruption of Kv2.1-VAPA association provides neuroprotection against ischemic stroke in mice by declustering Kv2.1 channels. Sci. Adv. 6, eaaz8110 (2020).