Excessive cytosolic calcium ion (Ca\(^{2+}\)) accumulation during cerebral ischemia triggers neuronal cell death, but the underlying mechanisms are poorly understood. Capacitive Ca\(^{2+}\) entry (CCE) is a process whereby depletion of intracellular Ca\(^{2+}\) stores causes the activation of plasma membrane Ca\(^{2+}\) channels. In nonexcitable cells, CCE is controlled by the endoplasmic reticulum (ER)–resident Ca\(^{2+}\) sensor STIM1, whereas the closely related protein STIM2 has been proposed to regulate basal cytosolic and ER Ca\(^{2+}\) concentrations and make only a minor contribution to CCE. Here, we show that STIM2, but not STIM1, is essential for CCE and ischemia-induced cytosolic Ca\(^{2+}\) accumulation in neurons. Neurons from Stim2\(^{-/-}\) mice showed significantly increased survival under hypoxic conditions compared to neurons from wild-type controls both in culture and in acute hippocampal slice preparations. In vivo, Stim2\(^{-/-}\) mice were markedly protected from neurological damage in a model of focal cerebral ischemia. These results implicate CCE in ischemic neuronal cell death and establish STIM2 as a critical mediator of this process.

**INTRODUCTION**

The intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) is a major determinant of the physiological state of all eukaryotic cells (1). In neurons, Ca\(^{2+}\) signals derived from intracellular stores or from the extracellular space are essential for various functions, including synaptic transmission and plasticity, but under pathological conditions, such as cerebral ischemia, a "calcium overload" can induce neuronal cell death (2–6). Two principal types of Ca\(^{2+}\) channels mediate Ca\(^{2+}\) entry into neurons: Voltage-gated Ca\(^{2+}\) channels and some ionotropic neurotransmitter receptors, including N-methyl-D-aspartate (NMDA) receptors (NMDARs) and some \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). Both of the latter are activated by the excitatory neurotransmitter glutamate, and glutamate excitotoxicity contributes to neurodegeneration in ischemia (4, 7, 8). Less is known about the presence and functional importance in neurons of store-operated Ca\(^{2+}\) (SOC) channels, which are activated in response to Ca\(^{2+}\) store depletion to allow capacitive Ca\(^{2+}\) entry [CCE, also called store-operated Ca\(^{2+}\) entry (SOCE)] and store replenishment (9, 10). STIM proteins are endoplasmic-sarcoplasmic reticulum (ER-SR)–resident Ca\(^{2+}\)-sensing molecules that regulate CCE (11). STIM1 is an essential component of CCE in different cell types, including lymphocytes (12–14), platelets (15), and (excitable) skeletal muscle cells (16), where it activates the SOC channel Orai1 (17) [also termed CRACM1 (18)] and possibly other SOC channels, whereas STIM2 has been proposed to regulate basal cytosol and store Ca\(^{2+}\) concentrations (19, 20), making a minor contribution to CCE in some cell types (14). No information, however, is available on the function of STIM proteins in neurons.

**RESULTS**

**STIM2 is the predominant STIM isoform in neurons**

We assessed STIM abundance in mouse brain by Western blot analysis and obtained a clear signal for STIM2, whereas STIM1 was only weakly detectable. We failed to observe a comparable predominance of STIM2 abundance in any other organ tested (Fig. 1A). Immunocytochemical analysis revealed hardly any signal for STIM1 in cultured hippocampal neurons, whereas strong perinuclear staining was detected with antibodies directed against STIM2, consistent with its expected ER localization (Fig. 1B). In contrast, in T cells the ratio of STIM1 to STIM2 abundance was reversed (Fig. 1B) (14). Reverse transcription–polymerase chain reaction (RT-PCR) analysis of primary hippocampal neurons isolated by laser capture microscopy confirmed that STIM2 was the predominant member of the STIM family present (Fig. 1C), indicating that it might have a role in Ca\(^{2+}\) homeostasis in these cells. Similar to Stim1, Orai1 messenger RNA (mRNA) was hardly detectable in neurons, whereas strong and weak expression was seen for the Orai1 homologs and putative STIM2 targets Orai2 and Orai3 (20), respectively (Fig. 1C). Williams and co-workers have previously shown that the mRNAs encoding both STIM1 and STIM2 are expressed in human brain samples, indicating that both proteins might be present in neuronal cells (21). We also detected Stim1 message in mouse brain tissue samples (Fig. 1C), and the protein is detectable in this organ (Fig. 1A). However, on the basis of data obtained with isolated neurons, it appears that these signals largely derive from cells other than neurons.
STIM2-deficient mice develop normally but have a reduced life expectancy

To assess STIM2 function in vivo, we disrupted the Stim2 gene in mice (Fig. 1, D and E). Mice heterozygous for the STIM2-null mutation were apparently healthy and had a normal life expectancy. Intercrossing of these animals yielded Stim2−/− mice at the expected Mendelian ratio, and the Stim2−/− mice developed normally to adulthood and were fertile (fig. S1). Western blot analyses confirmed the absence of STIM2 in the brain and lymph nodes (LNs), whereas STIM1 abundance was unaltered (Fig. 1F). Histological examination of major organs from Stim2−/− mice showed no obvious abnormalities. Starting at 8 weeks after birth, however, we observed sudden death of Stim2−/− mice, and only ~10% of the animals reached the age of 30 weeks (fig. S1). Spontaneous death of Stim2−/− mice has been reported by others (14), although in that study it occurred before adulthood (4 to 5 weeks after birth), a difference that may arise from differences in the genetic background of the two Stim2−/− mouse strains.

STIM2-deficient mice did not show any apparent neurological deficits, and Nissl staining of brain sections revealed no obvious structural abnormalities (fig. S2). However, a pronounced cognitive defect became apparent when the animals were subjected to the Morris Water Maze Task, the standard test for hippocampus-dependent spatial memory (22). Stim2−/− mice showed normal swimming ability but took longer to find the hidden platform, and the total distance moved was increased in acquisition trials, but not in reversal trials (Fig. 2A). In contrast, evaluation of anxiety-like behavior by the Elevated Plus Maze Task revealed no differences between Stim2−/− and wild-type littermates (P > 0.05; Fig. 2B). Thus, lack of STIM2 leads to a distinct cognitive impairment.

**STIM2 regulates CCE and ischemia-induced Ca2+ accumulation in neurons**

To test the effect of STIM2 deficiency on neuronal Ca2+ homeostasis directly, we performed Ca2+ imaging experiments in neuronal cultures...
extracted from cortical tissue. To assess CCE, we elicited Ca\(^{2+}\) store release by exposing cells to the sarcoendoplasmic reticulum Ca\(^{2+}\) adenosine-triphosphatase (SERCA) pump inhibitor cyclopiazonic acid (CPA) in the absence of extracellular Ca\(^{2+}\). This treatment caused a transient Ca\(^{2+}\) signal, followed by a second (Ca\(^{2+}\)) increase, after the addition of external Ca\(^{2+}\), that attained a peak signal that exceeded the basal Ca\(^{2+}\) concentration, suggesting the presence of CCE (Fig. 3A). CCE was reduced in Stim2\(^{-/-}\) neurons compared to that in wild-type controls (27 ± 9 nM versus 82 ± 16 nM; n = 7; P < 0.05; Fig. 3A), whereas no significant alterations in peak CCE were found in neurons from STIM1-deficient (15) and Orai1-deficient (23) mice compared to the respective controls (Fig. 3A). Thus, CCE in cultured neurons is regulated by STIM2 but does not require STIM1 or Orai1, the essential components of CCE in nonexcitable cells.

Brandman et al. have shown that STIM2 regulates basal Ca\(^{2+}\) concentrations in the cytosol and the ER of various nonneuronal cell lines (19). Consistent with this study, we found that Stim2\(^{-/-}\) neurons had lower basal Ca\(^{2+}\) than did wild-type neurons (62 ± 9 nM versus 103 ± 12 nM; n = 7; P < 0.05), whereas no such difference was seen in Stim1\(^{-/-}\) and Orai1\(^{-/-}\) neurons (Fig. 3A).

To evaluate the Ca\(^{2+}\) content of intracellular stores, we measured Ca\(^{2+}\) release from this compartment in response to application of the membrane-permeant calcium ionophore ionomycin in the absence of extracellular Ca\(^{2+}\) (19). The peak amplitude of Ca\(^{2+}\) release was decreased in Stim2\(^{-/-}\) neurons compared to that in control neurons (0.12 ± 0.02 versus 0.33 ± 0.07; n = 7; P < 0.05), whereas the subsequent Ca\(^{2+}\) entry after readdition of extracellular Ca\(^{2+}\) was indistinguishable (Fig. 3B). This observation is consistent with the notion that STIM2 regulates store refilling (11, 20), as well as the basal cytosolic Ca\(^{2+}\) concentration (19) in neurons by sensing ER Ca\(^{2+}\) content and, if it is too low, activating CCE. In wild-type neurons, the resulting Ca\(^{2+}\) influx will be used to refill Ca\(^{2+}\) stores. Consequently, ER Ca\(^{2+}\) content and the cytosolic Ca\(^{2+}\) concentration will be decreased in Stim2\(^{-/-}\) neurons compared to wild-type neurons.

During brain ischemia, excess [Ca\(^{2+}\)] is thought to play a critical role in triggering neuronal cell death (2). To evaluate the possible involvement of CCE in this process, we performed Ca\(^{2+}\)-imaging experiments on wild-type and Stim2\(^{-/-}\) neuronal cultures under conditions of oxygen-glucose deprivation (OGD), in some cases combined with a decreased pH (24), an established system for investigating calcium-dependent and calcium-independent mechanisms in neuronal injury (25, 26). OGD has been reported to lead to increased [Ca\(^{2+}\)], which is largely reversible when the duration of the insult is limited to 1 hour (26). We observed a robust increase in [Ca\(^{2+}\)] in the wild-type neurons (131 ± 27 nM; n = 5) but only a small increase in [Ca\(^{2+}\)] during 1 hour of OGD in Stim2\(^{-/-}\) cells (11 ± 15 nM; n = 5; P < 0.05; Fig. 3C). A marked increase in [Ca\(^{2+}\)] was apparent in Stim2\(^{-/-}\) cultures only when OGD was extended to 2 hours. The mitochondrial uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP, 2 μM, 30 min) elicited a large, rapid [Ca\(^{2+}\)] increase in both wild-type and Stim2\(^{-/-}\) neurons (Fig. 3D). However, after a 1-hour washout of CCCP, [Ca\(^{2+}\)] declined more rapidly and completely in Stim2\(^{-/-}\) neurons than in wild-type cells (89 ± 3% versus 72 ± 3%; n = 5; P < 0.05; Fig. 3D). Thus, ischemia-induced increases in [Ca\(^{2+}\)] develop more slowly and recover more rapidly in Stim2\(^{-/-}\) neurons compared to that in wild-type neurons, an observation that may, in part, be explained by the lower store content in these cells. It has been reported that anoxia causes slow depletion of the ER Ca\(^{2+}\) store and that SERCA plays a major role in cytosolic Ca\(^{2+}\) clearance in sensory neurons (27). Thus, a lower ER Ca\(^{2+}\) content would decrease the cytosolic Ca\(^{2+}\) load emanating from the ER and might also facilitate SERCA-mediated Ca\(^{2+}\) recovery under posts ischemic conditions.

The marked suppression of OGD-induced Ca\(^{2+}\) accumulation in Stim2\(^{-/-}\) neurons indicated a possible neuroprotective effect of STIM2 deficiency under ischemic conditions. To test this possibility directly, we subjected cultured hippocampal neurons to OGD (Fig. 4, A and B). After 5 to 7 days under control culture conditions, 80.6 ± 4.4% (n = 5) of wild-type neurons
Fig. 3. STIM2 regulates Ca\textsuperscript{2+} homeostasis in cortical neurons. (A) Neuronal cultures [5 to 9 days in vitro (DIV 5 to 9)] were loaded with fura-2 and averaged [Ca\textsuperscript{2+}] responses in [Ca\textsuperscript{2+}]\textsubscript{i} in the presence of EGTA. This Ca\textsuperscript{2+} release was normalized to the maximum response observed after replacement of 1 mM EGTA with 2 mM Ca\textsuperscript{2+}. Basal and peak [Ca\textsuperscript{2+}] were determined during the time intervals indicated. Store-operated [Ca\textsuperscript{2+}] increases (Δ[Ca\textsuperscript{2+}]) were calculated by subtracting basal [Ca\textsuperscript{2+}] from peak [Ca\textsuperscript{2+}]. (B) Ca\textsuperscript{2+} release from intracellular stores was elicited by the addition of 5 μM ionomycin in the presence of EGTA. This Ca\textsuperscript{2+} release was normalized to the maximum response observed after replacement of 1 mM EGTA with 2 mM Ca\textsuperscript{2+}. (C) Effect of combined OGD on [Ca\textsuperscript{2+}]\textsubscript{i}. Cultured neurons (DIV 12 to 14) were exposed for 1 hour (WT) or 2 hours (Stim2\textsuperscript{−/−}) to a glucose-free bath solution continuously bubbled with N\textsubscript{2}. The slight [Ca\textsuperscript{2+}] increase after about 70 min in the Stim2\textsuperscript{−/−} neurons occurred only in this particular experiment. (D) Chemical anoxia was induced in Stim2\textsuperscript{−/−} or WT cells by CCCP (2 μM, 30 min). Recovery from increased [Ca\textsuperscript{2+}]\textsubscript{i} was determined after washout of CCCP for 60 min. All bars represent means of five to seven experiments. Error bars indicate SEM. *P < 0.05, two-tailed Mann-Whitney U test.

were viable, in agreement with previous studies (28). There was a comparable number of viable Stim1\textsuperscript{−/−} neurons (76.7 ± 7.8%; n = 5; P > 0.05), whereas neurons prepared from Stim2\textsuperscript{−/−} mice showed increased viability (88.7 ± 2.6%; n = 3; P < 0.01). After 6 hours under ischemic conditions (low glucose, N\textsubscript{2}, pH 6.4, 7.25) showed 16.0 ± 1.7 dead neurons per square millimeter, whereas, in consecutive slices maintained under ischemic conditions, this number was increased (hypoglycemia, N\textsubscript{2}, pH 6.4, 30 ± 0.9; n = 3, P < 0.01). Compared to that in wild-type mice, fewer dead neurons were found in slices from Stim2\textsuperscript{−/−} mice under both control (7.7 ± 5.7, n = 3, P < 0.05) and ischemic conditions (18.6 ± 0.4, n = 3, P < 0.05; Fig. 4, A and F).

We performed experiments to discriminate among the effects of anoxia, low glucose concentration, and decreased pH on the survival of wild-type and Stim2\textsuperscript{−/−} neurons. Lowering the extracellular pH or glucose concentration reduced the survival of both wild-type and Stim2\textsuperscript{−/−} neurons compared to that in control conditions, although Stim2\textsuperscript{−/−} neurons survived both conditions significantly better than did wild-type cells. A strong protective effect of STIM2 deficiency was also apparent when the cells were exposed to anoxia alone or to anoxia in combination with low pH and reduced glucose concentration (fig. S3). In the next set of experiments, we assessed the role of STIM2 in other apoptosis-inducing pathways. Here, we treated cultured neurons with staurosporine, which modulates intracellular Ca\textsuperscript{2+} (29), or with N, N, N′, N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), which induces protein synthesis–dependent, caspase-11–mediated apoptosis that is largely Ca\textsuperscript{2+} independent (30). We found that Stim2\textsuperscript{−/−} neurons were protected from staurosporine (300 nM)-induced cell death compared to wild-type neurons (Fig. 4C), whereas TPEN (2 μM) induced apoptosis in wild-type and Stim2\textsuperscript{−/−} neurons to the same extent (Fig. 4D).

We also monitored neuronal death under ischemia-like conditions in mouse hemi-brain slices (Fig. 4, E and F). After 6 hours, slices from wild-type mice kept under control conditions (normoglycemia, O\textsubscript{2}, pH 7.25) showed 16.0 ± 1.7 dead neurons per square millimeter, whereas, in consecutive slices maintained under ischemic conditions, this number was increased (hypoglycemia, N\textsubscript{2}, pH 6.4, 30 ± 0.9; n = 3, P < 0.01). Compared to that in wild-type mice, fewer dead neurons were found in slices from Stim2\textsuperscript{−/−} mice under both control (7.7 ± 5.7, n = 3, P < 0.05) and ischemic conditions (18.6 ± 0.4, n = 3, P < 0.05; Fig. 4, E and F).

**Stim2\textsuperscript{−/−} mice are protected from ischemic stroke**

To determine the in vivo relevance of the above findings, we studied the development of neuronal damage in Stim2\textsuperscript{−/−} mice in response to 1-hour occlusion of the middle cerebral artery (MCA), a model of transient focal
cerebral ischemia (31). Twenty-four hours after MCA occlusion, infarct volumes in Stim2−/− mice were <40% the size of those in wild-type mice, as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining (18.6 ± 5.5 versus 57.9 ± 13.1 mm³, P < 0.01) (Fig. 5, A and B). The difference in infarct size was functionally relevant; the Bederson score assessing global neurological function was significantly better in Stim2−/− mice than in controls (1.6 ± 0.8 versus 3.0 ± 1.0, respectively; P < 0.05) (Fig. 5, A and B). The grip test, which measures motor function and coordination (2.0 ± 1.3 versus 4.1 ± 0.9, respectively; P < 0.05) (Fig. 5, C and D).

Serial magnetic resonance imaging (MRI) on living mice up to day 5 after MCA occlusion showed that infarct volume did not increase over time in Stim2−/− mice, indicating a sustained protective effect (fig. S4). Consistent with this, histological analysis revealed infarcts restricted to the basal ganglia in Stim2−/− mice, but extending into the neocortex in wild-type mice (Fig. 5E). Wild-type mice reconstituted with Stim2−/− bone marrow developed infarcts like those of control wild-type mice, whereas infarcts were small in Stim2−/− mice transplanted with wild-type bone marrow (Fig. 5, A to D). Thus, STIM2 deficiency protects mice
from ischemic neuronal damage independently of functional alterations within the hematopoietic system.

DISCUSSION

Our data show that STIM2 regulates CCE in neurons and that its absence from these cells leads to altered Ca\(^{2+}\) homeostasis. These data demonstrate that CCE, which is a well-established phenomenon in various cell types, is of (patho)physiological significance in cerebral neurons. The central role of STIM2 in this process was unanticipated given the essential role of STIM1 in CCE in the other cell types studied thus far (14–16). It is not clear why neurons should use STIM2 rather than STIM1 to regulate CCE, but differences between individual cell types in overall Ca\(^{2+}\) homeostasis may provide a possible explanation. Refilling of depleted intracellular Ca\(^{2+}\) stores, widely accepted as the foremost action of CCE in nonexcitable cells (9), may not be its main function in neurons where the continuous Ca\(^{2+}\) load associated with regular neuronal activity (32) should provide a sufficient supply of Ca\(^{2+}\). Furthermore, localized dendritic Ca\(^{2+}\) release events occurring during synaptic activity (33) are rapidly compensated for by shuttling of Ca\(^{2+}\) within the large continuous ER that extends to all parts of the neurons (3). However, the relative contribution of Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) entry to resulting Ca\(^{2+}\) signals may differ in functionally distinct neurons and may depend on the extent of synaptic activity. It is also conceivable that synaptic transmission produces a moderate but persistent deficit in ER [Ca\(^{2+}\)] because of release through ER inositol 1,4,5-trisphosphate (IP3) receptor and ryanodine receptor (RyR) channels. Whereas primary Ca\(^{2+}\) signals in hematopoietic (and other nonexcitable) cells involve brief but massive Ca\(^{2+}\) release and require rapid refilling of depleted intracellular stores, a more sustainable but lower-amplitude CCE may be needed in neurons. The apparently lower Ca\(^{2+}\) sensitivity of STIM2 compared with that of STIM1 leads to activation of STIM2 at a higher ER Ca\(^{2+}\) concentration, so that it is partially activated under basal conditions (19). Consequently, a reduced fraction of nonactivated STIM2 is available for mediating SOC channel activation, limiting the CCE signal. STIM1 and STIM2 have high sequence similarity (>65%) and an analogous structure, including the composition of the ER luminal Ca\(^{2+}\)-sensing domain responsible for STIM oligomerization and activation of ORAI channels (34, 35). However, the STIM2 oligomerization rate is markedly slower than that of STIM1 because of the stably folded state of the Ca\(^{2+}\)-sensing domain in STIM2 but not in STIM1 (37). The distinct oligomerization kinetics of the two STIM isoforms suggest that STIM2 elicits persistent CCE.

Fig. 5. Stim2\(^{-/-}\) mice are protected from neuronal damage after cerebral ischemia. (A to D) Wild-type and Stim2\(^{-/-}\) mice were subjected to tMCAO and analyzed after 24 hours. Experiments were also performed with wild-type mice transplanted with Stim2\(^{-/-}\) bone marrow (Stim2\(^{+/+}\)BM\(^{-/-}\)) and Stim2\(^{-/-}\) mice transplanted with wild-type bone marrow (Stim2\(^{-/-}\)BM\(^{+/+}\)). (A) Representative TTC stains of three corresponding coronal brain sections of the groups. Infarcted areas are indicated by arrows. (B) Brain infarct volumes as measured by planimetry at day 1 after tMCAO (n = 8 to 10 per group). (C) Neurological Bederson score and (D) grip test as assessed at day 1 after tMCAO. Graphs plot mean ± SD (n = 8 to 10 mice per group). (B to D) *P < 0.05, **P < 0.01, Bonferroni one-way ANOVA tested against wild-type mice. (E) Hematoxylin and eosin–stained sections in the ischemic hemispheres of wild-type (WT) and Stim2\(^{-/-}\) mice. Infarcts (white area) are restricted to the basal ganglia in Stim2\(^{-/-}\) mice, but consistently include the neocortex in the wild-type mice (10 of 10). Scale bars, 300 µm.
activation along with a small amplitude of Ca\(^{2+}\) entry, whereas STIM1 delivers a rapid, large-magnitude CCE signal.

The physiological roles of neuronal CCE may encompass unexpected functions, such as neurotransmitter release and synaptic plasticity (38). Indeed, we found impaired spatial learning in Stim2\(^{-/-}\) mice similar to that observed after blockade of NMDA-type ionotropic glutamate receptors (39). Further studies are required to draw a full picture of (i) the physiological role of STIM2 in neurons, (ii) the molecular partners with which STIM2 interacts (for instance, ORAI or TRP channels or both) (9, 20), (iii) the neuronal activity that stimulates CCE (for instance, burst firing or high-frequency discharges of excitatory inputs), and (iv) whether STIM2 deficiency affects synaptic transmission and plasticity.

Our study assigns a pathophysiological role to CCE in Ca\(^{2+}\)-dependent cell death, a process that evolves from or into glutamate-mediated neurotoxicity (6). Anoxia not only reduces SERCA activity in neurons (25, 27), but also appears to involve active Ca\(^{2+}\) release from IP\(_3\) receptor and RyR channels, as indicated by the ability of IP\(_3\) receptor or RyR blockade to protect neurons from excitotoxic injury (40, 41). Together, the reduction in SERCA activity combined with active Ca\(^{2+}\) release from the ER lead to Ca\(^{2+}\) accumulation in the cytosol and a corresponding store depletion, the latter eliciting an additional cytoplasmic Ca\(^{2+}\) load through CCE. In turn, CCE may trigger further Ca\(^{2+}\) influx by increasing the release of glutamate and the subsequent activation of ionotropic glutamate receptors (6). The combination of CCE and glutamatergic Ca\(^{2+}\) entry may then rapidly push cytosolic Ca\(^{2+}\) to dangerous concentrations. Neurons devoid of STIM2 may survive ischemic conditions better than wild-type neurons because they do not undergo CCE. Moreover, because Ca\(^{2+}\) stores rely on functional CCE (9), neurons lacking STIM2 can be expected, as an additional benefit, to have a lower store content. Decreased store content will limit the initial phase of Ca\(^{2+}\) release (see Fig. 3B) and enable the better utilization of the remaining Ca\(^{2+}\) sequestration capacity during an ischemic challenge.

In conclusion, our data establish Stim2 as a mediator of neuronal CCE and show that this pathway plays a critical role in hypoxia-induced neuronal death. Our findings may thus serve as a basis for the development of novel neuroprotective agents for the treatment of ischemic stroke and possibly other neurodegenerative disorders in which disturbances in cellular Ca\(^{2+}\) homeostasis constitute a major pathophysiological component (4, 5).

**MATERIALS AND METHODS**

**Mice**

Experiments were conducted in accordance with the regulations of the local authorities (Regierung von Unterfranken) and were approved by the institutional review boards of all participating institutions. The Stim2 knockout mouse was generated by deletion of most of exons 4 to 7 of the Stim2 gene by homologous recombination in R1 embryonic stem (ES) cells derived from the 129Sv mouse strain. Targeted stem cell clones were screened by Southern blot with a gene-specific external probe. Stim2\(^{-/-}\) ES cells were injected into a C57BL/6 blastocyst to generate chimeric mice. Chimeric mice were backcrossed with C57BL/6 (Harlan Laboratories) and subsequent progeny were intercrossed to obtain Stim2\(^{-/-}\) mice. Genotypes were determined by PCR analysis with the following primer pairs: Stim2 wild-type 5', CCCATATGTGAATGTTCCAG; Stim2 wild-type 3', GAGGTTGTTGT-CCTTCACAT; Stim2 knock-out 5', TTATCGATAGCGGCTGTTGTTAGC; Stim2 knock-out 3', GCGCTGATCCTGCGAATACATC.

Stim1\(^{+/+}\) and Orai1\(^{+/+}\) mice were generated as previously described (15, 23).

For the generation of bone marrow chimeras, 5- to 6-week-old Stim2\(^{+/+}\) and Stim2\(^{-/-}\) female mice were irradiated with a single dose of 10 Gy, and bone marrow cells from 6-week-old Stim2\(^{+/+}\) or Stim2\(^{-/-}\) mice from the same litter were injected intravenously into the irradiated mice (4 x 10\(^{6}\) cells per mouse).

**Western blots**

Western blot was performed with standard protocols on total protein lysates from different mouse organs extracted with radioimmunoprecipitation assay buffer Mill. The following primary antibodies were used for Western analysis: rabbit antibodies against STIM2-CT (1:400, ProSci) and STIM1 (1:500, Cell Signaling) and rat antibody against α-tubulin (1:1000, Chemicon International). All horseradish peroxidase-conjugated antibodies were purchased from Jackson Immunoresearch or Dianova. Bound antibodies were detected with enhanced chemiluminescent Western Lightning Plus-ECL (Perkin Elmer).

**RT-PCR analysis**

Mouse mRNA from 50 single hippocampal neurons isolated by laser capture microdissection was extracted and reverse-transcribed. Stim1 and Stim2 mRNA was detected by amplification of the 3' region (most heterogeneous region among STIMs) by RT-PCR with specific primers, as follows: Stim1/RT 5', CTTGCGGCTGGATCTCAGAG; Stim1/RT 3', TCAGC-CATTGCCCTTCTTGCC; Stim2/RT 5', GCAGAGCTTTCAGCCAGAC; Stim2/RT 3', ACATCTGCTGTCCAGGTTGA. Orai1 primers were as previously described (23).

**Histology**

Paraffin-embedded (Histolab Products AB) paraformaldehyde-fixed organs were cut into 5-μm-thick sections and mounted. After removal of paraffin, tissues were stained with hematoxylin and eosin (Sigma) or with the Nissl staining method according to standard protocols.

**Immunocytochemistry**

Hippocampal cell cultures were fixed with 4% paraformaldehyde (Merck, Germany), washed three times with 10 mM phosphate-buffered saline (PBS), and incubated for 60 min at 4°C in 10 mM PBS containing 5% goat serum (PAA Laboratories) and 0.3% Triton X-100. Primary antibodies (mouse antibody against MAP2a/b, 1:200, Abcam; rabbit antibody against cleaved caspase-3, 1:150, Cell Signaling) were diluted in 10 mM PBS and incubated for 12 hours at 4°C. The membranes were washed with 10 mM PBS and then incubated with secondary antibodies (Alexa 488-labeled goat antibody against mouse immunoglobulin G (IgG) 1:100, BD Bioscience; Cy-3-labeled goat antibody against rabbit IgG, 1:300, Dianova) in the same manner. Staining with 4',6'-diamidino-2-phenylindole (DAPI, 0.5 μg/ml, Merck) was performed for 7 min. Finally, cultures were washed and then covered with DABCO (1,4-diazabicyclo-[2,2,2]-octane, Merck). Images were obtained by immunofluorescence microscopy (Axioptot; Zeiss).

In one set of experiments, cultured neuronal cells as well as isolated murine CD4\(^{+}\) T cells from wild-type mice were placed on coverslips coated with poly-l-lysine (Sigma), fixed with 4% paraformaldehyde, and stained with antibodies against STIM1 (Cell Signaling) or STIM2 (Cell Signaling) antibodies followed by Cy-3-labeled goat antibody against rabbit IgG (Dianova). Cell nuclei were stained with DAPI (Merck).
Neuronal cell cultures

Neuronal cultures were obtained from *Stim1<sup>−/−</sup>, Stim2<sup>−/−</sup>, or control mice at E18 (embryonic day 18) according to previously described protocols (42). In brief, pregnant mice were killed by cervical dislocation and embryos were removed and transferred into warmed Hank’s buffered salt solution (HBSS). After the preparation of hippocampi, tissue was collected in a tube containing 5 ml of 0.25% trypsin in HBSS. After 5 min of incubation at 37°C, the tissue was washed twice with HBSS. Thereafter, the tissue was dissociated in 1 ml of neuronal medium by triturating with fire-polished Pasteur pipettes of decreasing tip diameter. Neurons were diluted in neuronal medium [10% 10<sup>−8</sup> modified Eagle’s medium (MEM), 0.22% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 2% B27 supplement (all Gibco); 3.8 mM glucose, 1% penicillin-streptomycin (Biochrom AG)] and plated at a density of 60,000 cells/cm<sup>2</sup> on poly-D-lysine–coated coverslips in four-well plates (Nunc). Before the experiments, all cell cultures were incubated at 37.0°C and 5% CO<sub>2</sub> and maintained in culture for up to 7 to 5 days. Cell viability was assessed by propidium iodide (57.14 ng/ml, Merck) staining and antibody staining for activated caspase-3 (1:200, Cell Signaling). For ischemic conditions, the pH was changed to 6.4, glucose concentrations were lowered to 5 mM, and O<sub>2</sub> was restricted as described for slice preparations. In additional sets of experiments, staurosporine (300 nM) or TPEN were added to the cell cultures.

For calcium measurements, primary neuronal cultures were obtained from *Stim1<sup>−/−</sup>, Stim2<sup>−/−</sup>, Orai1<sup>−/−</sup>, or control mice (E18 to postnatal day 0) as described above except for the following: Tissue was collected from whole cortices and cells were cultured in Neurobasal-A medium containing 2% B27 supplement, 1% GlutaMAX-I, and 1% penicillin-streptomycin (all Gibco). Cells were plated at a density of 50,000 cells/cm<sup>2</sup> on poly-D-lysine–coated coverslips in 24-well plates (Sarstedt) and were maintained in culture for up to 14 days.

Calcium imaging

Measurements of [Ca<sup>2+</sup>]<sub>i</sub> in single cortical neurons were carried out with the fluorescent indicator fura-2 in combination with a monochromator-based imaging system (T.I.L.L. Photonics) attached to an inverted microscope (BX51WI, Olympus). Emitted fluorescence was collected by a charge-coupled device camera. Cells were loaded with 5 μM fura-2-AM (Molecular Probes) supplemented with 0.01% Pluronic F127 for 35 min at 20°C to 22°C in a standard bath solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose, and 10 Heps adjusted to pH 7.4 with NaOH. For measurements of [Ca<sup>2+</sup>]<sub>i</sub>, cells were kept in a standard bath solution and fluorescence was excited at 340 and 380 nm. Fluorescence intensities from single cells were acquired at intervals of 2 or 20 s. After correction for the individual background fluorescence, the fluorescence ratio R = F<sub>340</sub>/F<sub>380</sub> was calculated. Quantities for [Ca<sup>2+</sup>]<sub>i</sub> were calculated with the equation [Ca<sup>2+</sup>]<sub>i</sub> = K<sub>D</sub>[βR − R<sub>min</sub>]/(R<sub>max</sub> − R), where K<sub>D</sub> = 224 nM, β = 2.64, R<sub>min</sub> = 0.272, and R<sub>max</sub> = 1.987, obtained from single dye-loaded cells in the presence of 5 μM ionomycin added to standard bath solution or to a solution containing 10 mM EGTA instead of 2 mM CaCl<sub>2</sub>.

Individual cells in a given culture dish showed variable kinetics but similar peak amplitudes of the Ca<sup>2+</sup> transients. Thus, for the average example traces illustrated in Fig. 3, the peak amplitudes, but not the kinetics, are comparable between dishes or cell types or both. In general, STIM2-mediated CCE signals were comparable to those in T cells and fibroblasts (14) but substantially smaller than those observed in other cell types, for example, HeLa cells (19).

For OGD experiments, cells were immediately transferred to a sealed, N<sub>2</sub>-purged (~2 liters/min) chamber continuously superfused with a N<sub>2</sub>-bubbled solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, and 10 Heps, adjusted to pH 7.4. All experiments were carried out at 20° to 22°C. All chemicals were obtained from Sigma.

Brain slice preparation

Brain slices including the hippocampus were prepared from 6- to 10-week-old *Stim2<sup>−/−</sup> mice and wild-type littermates as previously described (43). In brief, coronal sections were cut on a vibratome (Vibratome, Series 1000 Classic) in an ice-chilled solution containing (in mM) 200 sucrose, 20 Pipes, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 10 MgSO<sub>4</sub>, 0.5 CaCl<sub>2</sub>, and 10 dextrose; pH was adjusted to 7.35 with NaOH. After preparation, slices were transferred to a holding chamber continuously superfused with a solution containing (in mM) 120 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 30 Heps, 2 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>, and 10 dextrose. pH values were adjusted with HCl. To induce in vitro ischemic conditions, we reduced the pH from 7.25 to 6.4 and lowered the glucose concentration to 5 mM under hypoxic conditions. Restriction of O<sub>2</sub> was achieved by perfusion with an external solution that had been bubbled with nitrogen for at least 60 min before and throughout the recordings (24).

Morris Water Maze

The water maze consisted of a dark gray circular basin (120-cm-diameter) filled with water (24° to 26°C, 31 cm deep) that was made opaque by the addition of nontoxic white tempera paint. A circular platform (8-cm diameter) was placed 1 cm below the water surface in the center of the goal quadrant, 30 cm from the wall of the pool. Distant visual cues for navigation were provided by the laboratory environment; proximal visual cues consisted of four different posters placed on the inside walls of the pool. Animals were transferred from their cages to the pool in an opaque cup and were released from eight symmetrically placed positions on the pool perimeter in a predetermined but nonsequential order. Mice were allowed to swim until they found the platform or until 180 s had elapsed. In the latter case, animals were guided to the platform and allowed to rest for 20 s. The animals were submitted to six trials per day for 5 days, using a hidden platform at a fixed position (southeast) during the first 3 days (18 trials, acquisition phase) and in the opposite quadrant (northwest) for the last 2 days (12 trials, reversal phase). Trials 19 and 20 were defined as probe trials to analyze the precision of the spatial learning.

Elevated Plus Maze test

The animals were placed in the center of a maze with four arms arranged in the shape of a plus. Specifically, the maze consisted of a central quadrangle (5 × 5 cm), two opposing open arms (30 cm long, 5 cm wide), and two opposing closed arms of the same size but equipped with 15-cm high walls at their sides and the far end. The device was made of opaque gray plastic and was elevated 70 cm above the floor. The light intensity at the center quadrangle was 70 lux, in the open arms 80 lux, and in the closed arms 40 lux.

At the beginning of each trial, the animals were placed on the central quadrangle facing an open arm. The movements of the animals during a 5-min test period were tracked by a video camera positioned above the center of the maze and recorded with the software VideoMot2 (TSE Systems). After the test this software was used to evaluate the animal tracks and to determine the number of their entries into the open arms, the time spent on the open arms, and the total distance traveled in the open and closed arms during the test session. Entry into an arm was defined as the instance when the mouse placed its four paws on that arm.

Transient MCA occlusion model

Experiments were conducted on 10- to 12-week-old *Stim2<sup>−/−</sup> or control chimeras according to published recommendations for research in mechanism-driven basic stroke studies (44). Transient MCA occlusion...
(tMCAO) was induced under inhalation anesthesia with the intraluminal filament (6021PK10; Doccol Company) technique. After 60 min, the filament was withdrawn to allow reperfusion. For measurements of ischemic brain volume, animals were killed 24 hours after induction of tMCAO and brain sections were stained with 2% TTC (Sigma). Brain infarct volumes were calculated and corrected for edema as previously described (45). Neurological function and motor function were assessed by two independent and blinded investigators 24 hours after tMCAO as described (45).

Stroke assessment by MRI
MRI was performed repeatedly at 24 hours and 5 days after stroke on a 1.5-T MR unit (Vision Siemens) as described (45). MRI was performed at 24 hours and 5 days after tMCAO as described (45). Stroke assessment by MRI
SUPPLEMENTARY MATERIALS
The statistical methods used are given in the figure legends.

REFERENCES AND NOTES
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46. We thank M. Heckmann for helpful suggestions and critical discussion of the project. We are grateful to M. Boesl for performing the blastocyst microinjection. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Ni556/7-1 and SFB 688 TPA1, B1 to B.N. and G.S.) and the Rudolf Virchow Center. D.S. was supported by a grant from the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.
STIM2 Regulates Capacitive Ca^{2+} Entry in Neurons and Plays a Key Role in Hypoxic Neuronal Cell Death

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Resisting Ischemia

Loss of blood flow to the brain—as can occur during a stroke—leads to the death of neurons, a process that involves a pathological increase in intracellular calcium. Berna-Erro et al. investigated the role of capacitive calcium entry (CCE), a process in which depletion of calcium from intracellular stores triggers its entry across the plasma membrane, in ischemia-induced calcium entry and neuronal death. The calcium-sensing molecule STIM1 is known to play a crucial role in mediating CCE in various cell types; in neurons, however, Berna-Erro et al. found that CCE depended instead on the closely related molecule STIM2. Neurons from mice lacking STIM2 were resistant to the effects of hypoxia in vitro; moreover, mice lacking STIM2 showed less neurological damage than did wild-type mice in a model of ischemic stroke. Thus, the authors conclude that STIM2 is critical to neuronal CCE and that CCE plays a role in neuronal death in ischemia.