Nitric Oxide Modulates Spreading Depolarization Threshold in the Human and Rodent Cortex

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Background and Purpose—Recent clinical data have suggested that prolonged cortical spreading depolarizations (CSDs) contribute to the pathogenesis of delayed ischemic neurologic deficits after subarachnoid hemorrhage. Elevated extracellular potassium concentrations and lowered nitric oxide (NO) levels have been detected in experimental and clinical subarachnoid hemorrhage. We investigated whether a similar extracellular composition renders the brain more susceptible to CSDs.

Methods—Electrophysiologic and blood flow changes were studied in vivo in rats. Intrinsic optical signals, alterations of NO level, and electrophysiologic changes were investigated in rodent and human brain slices.

Results—Elevation of subarachnoid extracellular potassium in rats in vivo triggered CSDs. Using NO-sensitive dyes, we found that CSDs induce NO synthesis in neurons and endothelial cells. When we blocked NO synthesis in vivo, CSDs occurred at a significantly lower threshold and propagated with a wave of ischemia. This increased susceptibility for CSDs by a low NO level was confirmed in rat and human neocortical slices and depended on P/Q-type calcium channels and N-methyl-D-aspartate receptors, but not on guanylate cyclase. Mice deficient in endothelial NO synthase, in contrast to mice deficient in neuronal NO synthase, had an inherently lower threshold.

Conclusions—Basal NO production determined CSD threshold. The threshold effect depended predominantly on endothelial NO synthase. Reduced NO levels, as in patients with subarachnoid hemorrhage, may render the brain more susceptible to CSDs. Because CSDs have been linked to the pathogenesis of delayed ischemic neurologic deficits, raising its threshold by increasing NO availability may prove therapeutically beneficial in patients with subarachnoid hemorrhage. (Stroke. 2008;39:1292-1299.)

Key Words: subarachnoid hemorrhage ■ ischemia ■ nitric oxide ■ cortical spreading depolarization

Delayed ischemic neurologic deficits (DINDs) are a severe complication of subarachnoid hemorrhage (SAH). The risk for DINDs is correlated with the amount of subarachnoidal blood, and their appearance is time locked to the hemolysis, indicating a contribution of erythrocyte products.1

The Co-Operative Study on Brain Injury Depolarizations (COSBID), a prospective clinical multicenter study, demonstrated delayed clusters of cortical spreading depolarization (CSD) in SAH patients.4 In many patients, clusters of prolonged depolarization occurred. These patients developed ischemic infarcts in the recording area,4 indicating that CSDs may be linked to DINDs.

In rodents, the erythrocyte products hemoglobin and potassium, when applied in concentrations comparable to those found after cerebral hemorrhage,5,6 also trigger CSDs. These CSDs were accompanied by an ischemic change in regional cerebral blood flow (CBF), resulting in cortical infarcts (spreading ischemia).7 Because hemoglobin can be replaced by nitric oxide synthase (NOS) inhibitors, hemoglobin likely acts as an NO scavenger.8 Spreading ischemia appears to be pathophysiologically linked to DINDs, because (1) it is triggered by products of hemolysis7 in clinically relevant concentrations5,6 (2) spreading ischemia9,10 and DINDs11 share similar infarct morphologies, (3) extracellular NO is reduced after clinical and experimental SAH,12,13 and (4) nimodipine and hemodilution inhibit spreading ischemia14 and DINDs.15

These data indicate that prevention of CSDs in SAH may be useful in the treatment of DINDs. The human brain shows a much higher resistance to depolarizations compared with that of rodents.16 However, CSDs were surprisingly common in the COSBID study,4 indicating that unknown events after SAH increase the susceptibility to these depolarizations.

In the study reported herein, we investigated the mechanisms that decrease the threshold for spreading ischemia in the human and rodent brain. We demonstrated that NO, by
modulating neuronal ion channels, alters the threshold for depolarizations and that the threshold is mainly maintained by endothelial NOS (eNOS). Thus, pharmacotherapy aimed at increasing NO availability may prove therapeutically beneficial in the treatment of DINDs.

**Materials and Methods**

Detailed protocols are given in the supplemental Methods section available online at http://stroke.ahajournals.org.

**Brain Slice Experiments**

Coronal slices (400 μm) from rats (Charles River) or mice (Jackson Laboratories) were prepared as described and perfused with artificial cerebrospinal fluid (ACSF). CSDs were recorded by K⁺- selective/reference microelectrodes. Steady (direct current [DC]) potential amplitude, duration at half-maximal amplitude, and extracellular K⁺ concentration changes (\([K^+]_o\)) were analyzed. CSD was triggered either by slowly raising \([K^+]_{ACSF}\) or by microinjection of 3 mmol/L KCl.

Human brain tissue was obtained from patients undergoing surgery for pharmacoresistant epilepsy and were prepared as described. Slice viability was tested by recording field potentials in layers II/III after orthodromic stimulation in layer VI. Input/output curves were generated with increasing stimulation intensities. Intrinsinc optical signals were monitored and recorded as described. Slices were transilluminated and monitored with a CCD camera. Data were normalized to the first image in a series (\(ΔI/ΔT\%\)).

**Imaging NO Synthesis**

Slices were loaded in 1,2-diaminoanthraquinone (DAQ, Invitrogen) in ACSF (5 μg/mL). After the experiments, slices were fixed in 4% paraformaldehyde, cryosectioned for fluorescence microscopy, and analyzed with ImageJ software (National Institutes of Health, Bethesda, Md) as described.

For intracellular NO detection, slices were loaded with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM, 10 μmol/L; Invitrogen). After CSD, slices were fixed in 2% glutaraldehyde and cryosectioned. Sections were incubated with antibodies against glial fibrillary acidic protein (Dako), neuron-specific nuclear protein (NeuN, Chemicon), or von Willebrand factor antibodies against glial fibrillary acidic protein (Dako), neuron-glutaraldehyde and cryosectioned. Sections were incubated with their von Willebrand factor expression (Figures 2A through 2C) identified by their NeuN expression (Figures 2A through 2C), with 8% of neurons being DAF-positive. We also detected NO-related fluorescence in neurons, identified by their NeuN expression (Figures 2A through 2C). Secondary antibodies conjugated with Texas Red or Alexa selective/reference microelectrodes. Steady (direct current [DC]) potential amplitude, duration at half-maximal amplitude, and extracellular K⁺ concentration changes (\([K^+]_o\)) were analyzed. CSD was triggered either by slowly raising \([K^+]_{ACSF}\) or by microinjection of 3 mmol/L KCl.

**In Vivo Experiments**

Wistar rats were anesthetized with thiopental (BYK, 100 mg/kg IP) and intubated. The femoral artery and vein were cannulated for arterial pressure determination. Blood gases were measured serially. Closed cranial windows were implanted after craniotomy and duro removal as described and perfused with ACSF. CSD was monitored by 2 laser Doppler probes (Perimed). The DC potential was measured by an AgCl electrode. CSDs were elicited by increasing \([K^+]_{ACSF}\) by cathodal pulses in increasing intensity (10 to 400 μC; SD9 stimulator, Grass). NOS inhibition was achieved with N^ω-nitro-L-arginine (L-NNA; 2 mmol/L).

**Data Analysis**

Data were analyzed by comparing relative CBF and absolute DC potential values and \([K^+]_o\) changes. Input/output curves were analyzed by comparing evoked field potential amplitudes in response to multiples of stimulation threshold. Data are given as mean ± SD. P<0.05 was considered significant.

**Results**

**NO Production During CSD**

We used NO-sensitive fluorescent probes to investigate whether NO is produced during CSD in rat brain slices. CSD was triggered by local KCl microinjection, and slices were fixed for subsequent fluorescence microscopy.

Using the extracellular probe DAQ, we observed increased NO production after CSD (n=6; P<0.05, ANOVA followed by Tukey’s test) that occurred pericellularly in the neuropil (Figure 1A) and around blood vessels (Figure 1B). This increase was completely blocked by L-NNA (Figure 1C). In slices perfused with the neuronal NOS (nNOS) inhibitor 1-[2-(trifluoromethyl)phenyl]imidazole (TRIM; 100 μmol/L), NO-related fluorescence was relatively stronger around blood vessels (Figure 1D). The NO donor NO/ spermine strongly increased NO (100 μmol/L; Figure 1E). Under control conditions, low basal NO production occurred that was suppressed by the NOS inhibitor L-NNA (supplemental Figure I, available online at http://stroke.ahajournals.org). The data are summarized in Figure 1F. To determine the NO level during globally elevated \([K^+]_o\), just before CSD, we increased \([K^+]_{ACSF}\) to 15 mmol/L and fixed the slices. DAQ fluorescence significantly increased to 22 ± 4 compared with normal ACSF (P<0.05, Student’s t test). NO increased even further in contralateral slices, which were perfused with \([K^+]_{ACSF}\) until CSD occurred (33 ± 8; P<0.05, paired t test).

To determine the cellular sources of NO, we loaded slices with the cell-permeant dye DAF-FM. In areas invaded by CSD, we detected NO-related fluorescence in neurons, identified by their NeuN expression (Figures 2A through 2C). Neuronal processes could sometimes be identified (Figures 2D through 2F). No fluorescence occurred in areas not invaded by CSDs. The number of NeuN-positive neurons exceeded the number of NO-positive neurons (Figures 2C and 2F), with 8% of neurons being DAF-positive. We also detected signal in blood vessels (79% DAF-positive vessels). Most NO synthesis occurred in endothelial cells, identified by their von Willebrand factor expression (Figures 2G through 2I). We did not observe NO synthesis in astrocytes, identified by their glial fibrillary acidic protein expression (Figures 2J through 2L). In slices perfused with TRIM, the signal was restricted to the endothelium (Figures 2M through 2O).

**NOS Inhibition Lowers the CSD Threshold In Vivo**

We induced CSDs in vivo in rats by increasing \([K^+]_{ACSF}\). CSDs occurred at a \([K^+]_{ACSF}\) of 56 ± 8 mmol/L (n=8). When we blocked NOS with L-NNA in a separate group, CSDs occurred at a significantly lower threshold (30 ± 5 mmol/L; Figure 3A; P<0.001, Student’s t test, n=8).

As previously reported, an [NO] reduction changed the CBF response and duration of CSDs (Figures 3A and 3B). In brief, in CSDs elicited by potassium, depolarizations were blocked by NOS inhibition. The systemic variables remained within physiologic limits (the Table).

High \([K^+]_{ACSF}\) alone raised CBF to 154 ± 37%, compared with 90 ± 15% under high \([K^+]_{ACSF}\) and L-NNA. Therefore, to
control for possible vascular effects of potassium, we elicited CSDs by electric cortical stimulation before and after NOS inhibition. The CSD threshold was significantly lowered by L-NNA (n=5, Figure 3C; \(P<0.05\), paired t test), indicating that the effects of L-NNA unrelated to vasoconstriction contributed to the threshold effect.

NO Modulates the CSD Threshold in Rat and Human Brain Slices

Because NO can act on vessels and neurons, NO could decrease the threshold either by altering the CBF response to CSD or by nonvascular mechanisms. Therefore, we performed experiments in brain slices, which are devoid of CBF.

CSDs were triggered by increasing \([K^+]_{ACSF}\), and intracortical \([K^+]\), was measured. We detected CSDs at a \([K^+]\), of 15.6±1.7 mmol/L (Figure 4A). CSDs were characterized by a DC shift (−15.4±1.2 mV, 93±40 seconds), and a transient \([K^+]\), rise (42.9±6.9 mmol/L). In contralateral slices cocultured with L-NNA (1 mmol/L), CSDs occurred at a significantly lower threshold (Figure 4A, 10.8±1.1 mmol/L; \(P<0.05\), paired t test). Otherwise, the electrophysiologic features of CSDs did not differ (−12.3±2.4 mV, 76±25 seconds, \([K^+]\), 46.1±13.1 mmol/L). Field potential amplitudes and input/output curves after orthodromic stimulation were similar before and after L-NNA treatment (paired t test; Figure 4B), indicating that slice viability remained unaffected.

We also tested the effect of NO in human neocortical slices (n=6). The CSD threshold was 26.9±3.5 mmol/L (Figure 4C; −15.8±3.3 mV, 84±14 seconds, \([K^+]\), 49.9±6.0 mV). In slices treated with L-NNA, the threshold was significantly lower (20.8±3.7 mmol/L; \(P<0.05\), paired t test, Figure 4C). The electrophysiologic properties of CSDs remained unchanged (−18.3±3.1 mV, 79±21 seconds, \([K^+]\), 48.5±8.1 mmol/L; paired t test).

To monitor the propagation of CSDs, we recorded intrinsic optical signals. They were characterized by a decrease in light transmittance, propagating at 4.9±0.6 mm/min in controls and 5.1±0.7 mm/min in L-NNA–treated slices (\(P>0.05\), paired t test). In human brain slices, CSDs propagated at 4.2±0.7 mm/min (controls) and 4.7±0.9 mm/min (L-NNA; \(P>0.05\), paired t test). An example of CSDs in human tissue is given in supplemental Figure II.

NO Donors Revert the Threshold Effect

We coapplied the NO donor, NO/spermine (100 μmol/L), together with L-NNA to rat neocortical slices (n=6). The
CSD threshold was 16.1±2.2 mmol/L. The threshold in contralateral slices without L-NNa and NO/spermine was not different (14.8±1.3 mmol/L, paired t test; Figure 4A), indicating that NO donors can revert the threshold to control levels.

Endothelial NO Production Modulates the CSD Threshold

No significant threshold difference occurred when rat brain slices were treated with the nNOS inhibitor TRIM (100 μmol/L; 14.8±1.9 vs 15.2±3.0 mmol/L, paired t test), indicating that inhibition of NO synthesis by eNOS, or combined inhibition of nNOS and eNOS, confers the threshold effect.

To further investigate this issue, we determined the CSD threshold in mice deficient in eNOS or nNOS. In C57BL/6J mice (the genetic background strain), CSDs occurred at a [K⁺]e of 13.9±1.8 mmol/L (n=4, Figure 5A). The CSD threshold was similar in nNOS−/− mice (n=4) but significantly lower in eNOS−/− mice (n=4, Figure 5A; P<0.05, ANOVA followed by Tukey’s test). L-NNa lowered the CSD threshold in C57BL/6J and in nNOS−/− mice but not in eNOS−/− mice (Figure 5A). TRIM had no effect on all strains (Figure 5A). These data indicate that endothelial NO is predominantly responsible for the threshold effect.

CSD Threshold Modulation Depends on NMDA and P/Q-Type Calcium Channels

To investigate the role of cGMP, we applied the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (10 μmol/L) to rat brain slices (n=6). The CSD threshold was not significantly different (14.8±1.8 vs 15.1±2.1 mmol/L, paired t test).

Figure 2. CSDs trigger NO production in neurons and endothelium. Left, green fluorescence of the NO-sensitive probe DAF-FM; middle, secondary antibodies. Colocalized signals appear yellow in composite images (right). A and B, NO production in neurons, identified by their NeuN expression. C, Only some neurons (arrowheads) produced NO during spreading depolarization. D–F, Major neuronal processes were positive for DAF-FM fluorescence (arrow). G–I, NO production in endothelial cells (arrowhead) was identified by their von Willebrand factor expression. J–L, No colocalization was seen with astrocytes, identified by glial fibrillary acidic protein expression (arrowheads, positive neurons). M–O, The nNOS inhibitor TRIM suppressed NO production in neurons (NeuN, blue), but not in the endothelium (von Willebrand factor, red). Scale bars=10 μm.
To investigate the actions of NO upstream of guanylate cyclase, we investigated the role of voltage-gated calcium channels and N-methyl-D-aspartate (NMDA) receptors. These channels are directly modulated by NO, and they mediate calcium elevations during CSD. Rat brain slices were perfused with the N-type channel antagonist ω-conotoxin GVIA (2 μmol/L, n = 6), the L-type antagonist nimodipine (5 μmol/L, n = 6), the P/Q-type antagonist ω-agatoxin IVA (300 nmol/L, n = 6), or the NMDA receptor antagonist MK-801 (20 μmol/L, n = 6). The CSD threshold was higher in slices perfused with calcium channel antagonists compared with slices in which CSDs were triggered by elevated [K⁺]ACSF (see previous section and Figure 5B; P < 0.05, Student’s t test).

Contralateral hemispheres in all groups were perfused with L-NNA in addition to the antagonists. In slices perfused with ω-conotoxin GVIA and nimodipine, the threshold modulation by L-NNA still existed (P < 0.05, paired t test; Figure 5B), whereas no significant differences between L-NNA–treated slices and controls were observed in groups perfused with ω-agatoxin IVA or MK-801 (paired t test; Figure 5B), indicating that NO acts upstream of guanylate cyclase by P/Q-type channel and NMDA receptor modulation. All drugs reduced evoked field potential amplitudes (supplemental Figure III), indicating sufficient tissue concentrations.

Discussion

DINDs represent a severe complication after SAH. They are time locked to the hemolysis of the subarachnoid blood clot, indicating erythrocyte products as triggering factors. Likely candidates are hemoglobin and potassium, which are very abundant in erythrocytes and are both released into the subarachnoid space during hemolysis. Hemoglobin scavenges NO, indicated by the decline of NO levels in patients with DINDs. Markedly increased extracellular potassium levels occur over several days in the blood clot in patients with cerebral hemorrhage, with a time course matching that of hemolysis. Intracortical potassium elevations have also been detected in experimental SAH.

We have previously shown that the combination of low NO and elevated potassium induces neocortical depolarizations that propagate with an ischemic CBF wave (spreading ischemia). Spreading ischemia induces laminar cortical necrosis, a lesion pattern also characteristic of DINDs after SAH. The clinical relevance of this concept is supported by recent findings of a status of recurrent prolonged spreading depolarizations in SAH patients developing DINDs. It has remained undetermined why the brains of these patients are more susceptible to CSDs. This question would seem espe-

Figure 3. Reduction of NO levels lowers the CSD threshold in vivo. A, CSDs were elicited by applying increasing concentrations of potassium in the ACSF to the cortex (black bars). It was accompanied by transient hyperemia. Coapplication of potassium with the NOS inhibitor L-NNA inverted the CBF response to CSD to ischemic levels (spreading ischemia, white bars) and induced a significant prolongation of the depolarization period. Compared with CSDs in the absence of NOS inhibition, spreading ischemia occurred at a significantly lower threshold (Student’s t test, *P < 0.001), indicating that the CSD threshold was lowered by low NO levels in vivo. B, Recordings of CBF and DC potential during spreading ischemia triggered by potassium and L-NNA and CSDs triggered by potassium alone (ie, in the absence of NOS inhibition). DC amplitude was defined as the largest deflection from baseline (0 mV, dashed line) during depolarization (a). The DC shift duration was defined as the duration of the negative DC deflection at half-maximal amplitude (b). During spreading ischemia, CBF decreased to ischemic levels (amplitude [c], lowest CBF level relative to baseline [−100%; dashed line]), followed by mild hyperemia. During CSDs triggered by potassium alone, CBF showed a mild and variable hypoperfusion that was followed by strong hyperemia (d). The ischemic CBF change and the depolarization during spreading ischemia were significantly prolonged (> 20 minutes) compared with CSD in the absence of NOS inhibition.

C, CSD triggered by electric stimulation before and after L-NNA application. NOS inhibition significantly lowered the threshold (*P < 0.05, paired t test).
cally critical, as elucidation of underlying mechanisms could lead to new therapeutic strategies for DINDs.

In this study, we addressed this issue by studying CSDs in vivo and in vitro. We found that a decline of the tissue NO concentration profoundly lowered the CSD threshold. Modulation of the CSD threshold by L-NNA in slices, the effect of P/Q-type channel and NMDA receptor blockers, and the existence of threshold modulation by L-NNA in electrically elicited CSDs indicate that direct neuronal effects of NO on the threshold predominate over vascular effects. Importantly, our data suggest that the CSD threshold is determined by basal NO levels and not directly by the large NO surge after CSD.

However, the generally higher CSD threshold in vivo compared with slices indicates that vascular factors, eg, impaired vascular K⁺ buffering induced by L-NNA, also contribute to the threshold. Moreover, when CSD was elicited by high [K⁺]ACSF in vivo, NOS inhibition increased CSD duration, as reported. Interestingly, this effect of L-NNA on CSD duration was not apparent in brain slices, indicating that, contrary to some reports, prolongation of the CSD by NOS inhibition in vivo is mainly a vascular rather than a direct neuronal effect.

When CSD is triggered by application of potassium alone, its threshold in the human cortex is high. Consequently, if the mean potassium concentrations measured in patients with cerebral hemorrhage would have been applied to human brain slices in our model, CSDs would likely have not occurred in most experiments. However, because of the threshold reduction by low NO levels, the subarachnoid potassium concentration necessary to trigger CSDs in human and rodent brains in our model matched the concentrations found in patients with cerebral hemorrhage remarkably well.

A potential, but unavoidable, caveat is that the human tissue in our study was derived from patients with intractable epilepsy.

These results suggest that a decline of NO may be a clinically relevant risk factor for CSDs in SAH patients. NO modulates neuronal ion channels, but it also acts on guanylate cyclase. Our data indicate that the threshold is predominantly determined by NO acting on P/Q-type calcium channels and NMDA receptors. NO likely modulates these channels directly, because blockade of guanylate cyclase had no effect. Direct modulation of these channels by NO via nitrosylation is well documented.

The CSD threshold in wild-type and nNOS−/− mice was similar to that in rat brain slices and was reduced by L-NNA but not by TRIM. In contrast, eNOS−/− mice had an inherently lower threshold that was unresponsive to L-NNA or TRIM. Specificity and adequate drug levels of TRIM were confirmed by the selective blockade of DAF-FM fluorescence in neurons. These results indicate that a normal CSD threshold can be maintained by endothelial NO production alone and that the threshold effect is mainly mediated by eNOS. Given the small distance between capillaries and neurons and the long range of NO signaling, endothelial NO is able to reach the sites required for the threshold effect. This diffusibility was also apparent in the DAQ experiments, but surprisingly, not in the DAF-FM experiments. This may be related to the adverse effects of fixatives on DAF-FM fluorescence.

### Table. Physiologic Variables

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<th>Mean Arterial Blood Pressure, mm Hg</th>
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<th>PaO₂, mm Hg</th>
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<td>Potassium (n=8)</td>
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<td>109±8</td>
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<td>Potassium and L-NNA (n=8)</td>
<td>94±17</td>
<td>38.2±4.9</td>
<td>110±9</td>
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Table 4. NO modulates the threshold for CSDs in rat and human brain slices. A, L-NNA significantly reduced the threshold in rat slices, indicating effects unrelated to vascular changes. The effect was reverted by the NO donor NO spermine (paired t test, **P<0.001**). B, Input/output curves of evoked field potentials recorded at the end of experiments in layers II/III to stimulation between layer VI and white matter. The stimulation threshold was defined for each slice as the minimum present required to elicit a field potential. Stimulus intensity is given as threshold multiples (×T). Responses are normalized relative to maximal amplitude. Open circles, L-NNA; closed circles, contralateral control slices. C, In the human neocortex, L-NNA significantly reduced the threshold compared with neighboring slices perfused with physiologic ACSF (paired t test, #P<0.05). The absolute threshold was higher in human tissue compared with rat brain, as previously reported.
An additional finding is that if CSD is triggered in the absence of NO-lowering substances, relatively large amounts of NO are produced by neurons and endothelial cells. Although basal NO levels, rather than this increase, determine the CSD threshold, the instantaneous release of NO by these cells may constitute an endogenous negative-feedback loop that protects the brain from recurrent CSDs.

In conclusion, we have shown that the likelihood of deleterious depolarization events increases when NO production in the brain is compromised. Given the likely link between these depolarizations and DINDs, substances that increase NO, interfere with NO-lowering mechanisms, or act on the targets of NO may be useful in the prophylaxis and treatment of DINDs.

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References


None.

Figure 5. Predominant modulation of CSD threshold by eNOS, NMDA receptors, and P/Q-type calcium channels. A, Mice deficient in eNOS or nNOS were compared with C57BL/6J mice (their genetic background strain). Wild-type and nNOS-/- mice had comparable CSD thresholds, whereas eNOS-/- mice had significantly lower thresholds. L-NNA significantly lowered the CSD threshold in wild-type and nNOS-/- mice but not in eNOS-/- mice. Selective nNOS inhibition with TRIM had no effect on the threshold in all strains (P<0.05, ANOVA followed by Tukey’s test). B, Rat brain slices were perfused with the N-type channel antagonist ω-conotoxin GVIA, the L-type antagonist nimbodipine, the P/Q-type antagonist ω-agatoxin IVA, or the NMDA receptor antagonist MK-801. The effect of L-NNA on the threshold for CSD was determined in these slices (black bars) and compared with contralateral control slices (white bars). The lowered threshold induced by L-NNA still existed in slices perfused with N-type and L-type calcium blockers but was abolished in slices perfused with P/Q-type and NMDA receptor antagonists (paired t test, *P<0.05).


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