Cortical spreading depression-induced preconditioning in mouse neocortex is lamina specific

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Cortical spreading depression-induced preconditioning in mouse neocortex is lamina specific. J Neurophysiol 109: 2923–2936, 2013. First published March 20, 2013; doi:10.1152/jn.00855.2011.—Cortical spreading depression (CSD) is able to confer neuroprotection when delivered at least 1 day in advance of an ischemic event. However, its ability to confer neuroprotection in a more immediate time frame has not previously been investigated. Here we have used mouse neocortical brain slices to study the effects of repeated episodes of CSD in layer V and layer II/III pyramidal neurons. In layer V, CSD evoked at 15-min intervals caused successively smaller membrane depolarizations and increases in intracellular calcium compared with the response to the first CSD. With an inter-CSD interval of 30 min this preconditioning effect was much less marked, indicating that preconditioning lasts between 15 and 30 min. A single episode of CSD also provided a degree of protection in oxygen-glucose deprivation (OGD) by significantly shortening the lifetime a cell could withstand OGD before anoxic depolarization occurred. In layer II/III pyramidal neurons no preconditioning by CSD on subsequent episodes of CSD was observed, demonstrating that the response of pyramidal neurons to repeated CSD is lamina specific. The A1 receptor antagonist 8-cyclopentyl theophylline (8-CPT) reduced the layer V preconditioning in a concentration-related manner. Inhibition of extracellular formation of adenosine by blocking ecto-5′-nucleotidase with α,β-methyleneadenosine 5′-diphosphate prevented preconditioning in most but not all cells. Block of equilibrative nucleoside transporters 1 and 2 with dipyridamole alone or in combination with 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine also prevented preconditioning in some but not all cells. These data provide evidence that rapid preconditioning of one CSD by another is primarily mediated by adenosine.

Cortical spreading depression; adenosine; preconditioning;

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Nedergaard and Hansen 1988). Interestingly, when a single CSD is induced by needle prick, brief electrical stimulation, or brief KCl application, either in vivo or in vitro, a “relative refractory period” for CSD has been noted (Bures et al. 1974; Walz 1997). During this period it is harder to elicit another CSD; this effect has generally been put down to the need for a critical mass of neurons to recover their excitability after the ionic and synaptic disturbances of CSD.

However, other mechanisms may be at play. For example, a reduction in the amplitude of a second CSD elicited 30 min after a first has been noted (Stone 2000), and this effect was attenuated by chlormethiazole. However, since the exact mechanism by which the drug exerted this effect was not clear, this result throws no specific light on the mechanism contributing to the smaller second CSD.

It may be helpful to consider the result in terms of preconditioning whereby a first CSD has a preconditioning effect on a later CSD, and possibly also on later ischemic events. Indeed, a decrease in lesion size when CSD is delivered at least 1 day in advance of an ischemic event does occur (Kawahara et al. 1999; Kobayashi et al. 1995; Matsushima et al. 1996; Yamanoto et al. 2004). However, there has been no investigation of whether CSD is able to induce preconditioning on a more rapid timescale—on the order of 5–10 min—similar to that seen with ischemic preconditioning (IPC) in heart and brain (Murry et al. 1986; Stagliao et al. 1999). The mechanism underlying the rapid neuroprotection conferred by IPC involves adenosine, acting on the A1 receptor, as the key modulator (Heurteaux et al. 1995; Liu et al. 1994; Perez-Pinzon et al. 1996; Puligiese et al. 2003).

In the neocortex and hippocampus a tonic level of adenosine is present and it persistently suppresses synaptic transmission via activation of A1 receptors (Dunwiddie 1980). Much of this adenosine appears to be derived from extracellular degradation of astrocytically released ATP (Pascual et al. 2005), although adenosine release from either neurons or glial cells by equilibrative transporters or vesicular mechanisms provides other sources of extracellular adenosine (Sperlagh and Vizi 2011). Glutamatergic activity-dependent heterosynaptic depression also occurs as a result of glial ATP release (Zhang et al. 2005) and its degradation to adenosine with consequent activation of the A1 receptor (Pascual et al. 2005). Given the widespread distribution of A1 receptors in the neocortex and hippocampus (Fredholm et al. 2001) and the fact that the depolarization of CSD initially involves strong neuronal firing (Gniel and Martin 2010), it seems feasible that release of ATP and its degradation to adenosine will occur in this condition. As a consequence, CSD-induced preconditioning on a rapid timescale could be expected.
In this study we used mouse neocortical slices to examine the neuronal responses to repeated CSD induced by brief superfusion of 26 mM KCl in healthy tissue. We have also determined whether CSD modulates, and is modulated by, oxygen-glucose deprivation (OGD), an in vitro model of ischemia. Our data indicate that in layer V, but not in layer II/III, CSD exerts rapid and short-lasting preconditioning on itself and to OGD. In addition, brief prior OGD limits the neural response to repeated CSD. As in the heart, adenosine, acting on the A1 receptor, plays an important role in these responses. The extracellular adenosine appears to be derived from an interplay of enzymatic degradation of AMP and transmembrane movement via equilibrative nucleoside transporters (ENTs).

MATERIALS AND METHODS

Animals and slice preparation. Male FVB/n transgenic (GFAP-GFP; Jackson Laboratories) or wild-type FVB/n mice aged 16–26 days were used for this study. Although astrocytes of these mice fluoresce under the appropriate light, we did not image astrocytes at all. Animals were maintained in a 12:12-h light-dark cycle and given free access to food and water. The use of animals was approved by the Australian National University Animal Ethics and Experimentation Committee. Mice were killed by rapid decapitation without anesthesia (NEMI Scientific small animal guillotine), and the brain was quickly removed in ice-cold modified artificial cerebrospinal fluid (ACSF) containing (mM) 125 NaCl, 3 KCl, 23 NaHCO3, 1.25 Na2HPO4, 25 glucose, 0.5 CaCl2, 6 MgCl2, and 1.75 ascorbate and continuously bubbled with 95% O2-5% CO2. Three hundred-micrometer-thick coronal slices containing hippocampus were prepared with a Vibratome 1000 Plus. Slices were transferred to an incubation chamber held at 34°C and containing standard ACSF with the following composition (mM): 125 NaCl, 3 KCl, 23 NaHCO3, 1.25 Na2HPO4, 25 glucose, 2 CaCl2, 1 MgCl2, 3 pyruvate, and 2 ascorbate. Slices were incubated for 45 min and then allowed to cool to room temperature for at least 30 min prior to use. Individual slices were transferred to a perfusion chamber (volume ∼1 ml) on the stage of an upright microscope (Olympus BX50W1) and held in place with a platinum wire. Slices were superfused with standard ACSF at a rate of 4–4.5 ml/min. Bath temperature was maintained at 34 ± 0.5°C with an in-line heater (Warner Instruments; model TC-324B).

Electrophysiological recordings. Layer V and layer II/III pyramidal neurons located in the primary motor cortex were identified visually. Patch electrodes with a resistance of 2.5–5.5 MΩ (layer V) or 4.5–7.5 MΩ (layer II/III) were pulled from borosilicate glass with a wall thickness of 0.5 mm (Hilgenberg, Germany) with a Flaming/Brown micropipette puller (Sutter Instruments; model P-87). The patch-clamp technique was used in whole cell mode to deliver the cell-impermeant form of the ratiometric calcium dye fura-4F (Molecular Probes, Eugene, OR). The Kd of fura-4F has been reported as 1.0 μM (Ismailov et al. 2004) and 1.1 μM (Felny et al. 2003); if an average Kd of 1.05 μM is assumed, then Cα2+ concentrations in the range of 100 nM to 10 μM may reliably be measured (Haugland 1992). The intracellular solution contained (mM) 115 K-glucuronate, 20 KCl, 8 NaCl, 10 HEPES, 1 Na2ATP, 0.3 NaGTP, 2 MgCl2, and 0.05 fura-4F. Current-clamp recordings commenced 20 min after breakthrough into whole cell recording mode. Initially, the spiking characteristics of the neuron were digitized at 2 kHz with a Digidata 1322A (Molecular Devices) and recorded with pCLAMP software (version 9.0; Molecular Devices). Thereafter, responses to solution changes were digitized at 2 Hz. A DC recording electrode (2.5–7.5 MΩ; filled with 2 M NaCl) was placed ∼750 μm from the patch electrode in layer II/III of the cortex. Membrane potential, as reported in RESULTS, was measured as the difference between the voltage recorded on the DC electrode and the patch electrode. Although the DC recording electrode was not immediately adjacent to the patch electrode, which could potentially result in a different field potential at the site of the extracellular electrode during CSD, any effect is likely to be negligible because of the comparatively large change in the intracellular potential.

Measurement of DC potential amplitude. The baseline measurement used in determination of the amplitude of the DC shift contained five points (recorded at the sampling rate of 2 Hz) from immediately before the DC deflection began. The abruptness of the DC shift made it apparent which points to use. Three points were averaged at the peak of the DC shift; subtracting the average of the baseline points gave the amplitude of the DC shift.

Voltage step protocol. The command resting membrane potential was set at −70 mV for all voltage-clamp experiments and depolarizing voltage steps to −30 mV or +5 mV were made in some experiments. Series resistance (Rs) ranged from 6 to 16 MΩ (average = 9 ± 0.5 MΩ) with a minimum of 75% compensation (average = 80 ± 0.5%). When the command voltage was −30 mV the average current amplitude was 0.9 nA, giving a small voltage correction of 1.6 mV. However, a command voltage of +5 mV resulted in an average current of 11.1 nA. This is associated with a voltage correction of 19.4 mV. As a result, the membrane potential that the cell would have been held at is estimated at −15 mV. The corrected voltages, rather than command voltages, are given in this report.

Calcium imaging. Fura-4F was alternately excited at 340 and 380 nm with a TILL Polychrome IV monochromator (TILL Photonics) fitted with a 150-W xenon lamp light source. Images were acquired with a submerged ×40 objective with a PCO Sensicam CCD camera and TILL Vision software (version 4.0). Each ratio set also contained an image taken at the isosbestic wavelength (352 or 354 nm). An exposure time of 10 ms was used at each wavelength, resulting in a total exposure time of 30 ms per measurement. Electrophysiological recording was initiated at time zero on a master clock, and times of image collection were recorded from this master clock. This means that alignment of the two types of data is accurate to within ~1 s. The significant cell swelling that occurs in the wake of the depolarization associated with CSD and OGD required regular refocusing of the image, and therefore images were taken intermittently rather than at preset intervals.

Analysis of calcium imaging data. The data were analyzed in the same manner as we reported previously (Gniel and Martin 2010). Briefly, images were analyzed off-line with TILL Vision software (version 4.0). At each time point a region of interest (ROI) was drawn around the soma, immediately within the fluorescence boundary, and designated ROIcell. As single cells were filled with a patch pipette, there was a clear demarcation between the cell and the background. Redrawing of the ROI at each time point meant that any changes to cell size or position as a result of CSD-associated cell swelling were corrected for. To correct for background fluorescence, a second ROI was drawn close to the cell (ROIbackground). The average pixel intensity of ROIbackground was subtracted from each pixel in ROIcell. Threshold limits were set for each wavelength (340 nm = 10; 380 nm = 7), and values below these limits were clipped to zero. For all experiments, average pixel intensity for the image at 340 nm plotted against calcium concentration showed that if the average pixel intensity fell below 60 it no longer correlated with calcium concentration. Therefore, data were discarded if fluorescence decreased below this level.

Maximum ratio (Rmax) was determined by using the patch electrode to equilibrate the cell with a very high calcium concentration (50 mM), as has been described previously (Schiller et al. 1995). Minimum ratio (Rmin) was similarly determined by equilibration of the cell with an intracellular solution containing 10 mM KEGTA. Rmax and Rmin were determined for each batch of fura-4F, in a minimum of four cells; β was determined for each batch of fura-4F and then further refined for each cell in accordance with the method outlined by Gomes et al. (1998).

Induction and confirmation of cortical spreading depression. CSD was induced by a 2-min bath application of a modified ACSF containing...
containing 26 mM KCl substituted for equimolar NaCl, as has been previously described (Anderson and Andrew 2002). This protocol induced CSD in all slices. CSD was confirmed by the presence of a negative shift in the DC potential that propagated, in either direction, between the DC electrode and the patch electrode (see Gniel and Martin 2010). Repeated episodes of CSD were induced at 15-min intervals, thereby avoiding the post-CSD period of absolute refractoriness (lasting ~2 min; Herrera and Somjen 1993) and the period of relative refractoriness (lasting up to 10 min; Bures et al. 1974). However, as reported previously (Anderson and Andrew 2002), it was still necessary to raise the rate of perfusion to >4 ml/min to elicit repeated CSD in the submerged slices. Even so, a second DC shift only occurred in 22 of the 30 slices in which attempts were made to elicit CSD. Only 16 of these 22 slices displayed a third consecutive DC shift. Continuous recordings of membrane potential and the extracellular DC potential continued during the inter-CSD interval. Measurements of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{i}) were made to confirm that it remained at the low level it had returned to after an initial episode of CSD; however, these measurements were infrequent so as to limit bleaching of fura-4F.

Applicaton of oxygen-glucose deprivation. To investigate the effects of CSD in metabolically compromised neurons, slices were subjected to OGD by changing the perfusate from standard ACSF to one in which glucose had been replaced with mannitol and that was bubbled with 95\% N\(_2\)-5\% CO\(_2\) (referred to as OGD ACSF).

The duration of the OGD exposure was governed by the hyperpolarization that typically precedes anoxic depolarization (AD; described in Gniel and Martin 2010). Once a hyperpolarization of 2 mV was observed, CSD was induced by switching to the CSD-initiating ACSF for 2 min. After this time period, the perfusate was switched back to the standard ACSF, and a second episode of CSD was initiated 15 min after the first. After repolarization from the second episode of CSD, the perfusate was once again changed to OGD ACSF to induce the AD.

Drugs. To chelate intracellular Ca\(^{2+}\) the intracellular solution contained 20 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA tetrapotassium salt, Sigma). The K-glucuronate concentration was lowered to 35 mM to maintain a K\(^{+}\) concentration of 115 mM. The A1 receptor antagonist 8-cyclopentyl theophylline (8-CPT, Sigma) was prepared as a 40 mM stock solution dissolved in 1 M NaOH and stored at −20°C. The stock solution was diluted to a final concentration of either 1 \(\mu\)M or 3 \(\mu\)M in standard ACSF solution. The pH of the ACSF solution was not altered by the addition of 8-CPT. The reversible NMDA receptor antagonist d(-)2-amino-5-phosphonopentanoic acid (APV, Sigma) was dissolved in H\(_2\)O to give a 50 mM stock solution, which was frozen and diluted in ACSF on the day of use to give a final concentration of 75 \(\mu\)M in the perfusate. Transport of adenosine through ENTs was blocked with dipyridamole (DIPY, Sigma) alone or with 6-[(4-nitrobenzyl)thio]-9-\(\beta\)-ribofuranosylpurine (NBTI, Sigma). A frozen 1,000× stock of DIPY was dissolved in DMSO and thawed and diluted in ACSF on the day of use, to give a final concentration of 10 \(\mu\)M DIPY and 0.1% DMSO. When NBTI was used with DIPY they were both dissolved in DMSO to give a 1,000× stock solution of each and diluted in ACSF on the day of use, to give a final concentration of 10 \(\mu\)M DIPY, 5 \(\mu\)M NBTI, and 0.1% DMSO.

Statistical analysis. Results are presented as means ± SD. The statistical test used for each analysis varied depending on experimental design and is given in parentheses alongside the P value. P values ≤ 0.05 were considered significant. When a comparison was made between two groups and all data were collected from different cells, an unpaired Student’s t-test was used. Where data were collected across the three times CSD was induced or across three drug concentrations, an analysis of variance (ANOVA) was employed. Where the statistical test is listed as a “repeated-measures analysis” this refers to a linear mixed-effect model (GenStat version 14). It was used to identify treatment (drug) effects across the three times at which CSD was induced.

RESULTS

Repeated episodes of CSD in layer V pyramidal neurons. Induction of CSD by high-K\(^+\) solution caused an initial K\(^+\)-induced depolarization to about −30 mV followed by a second much more rapid depolarization to about 0 mV that was accompanied by a sharp shift in the DC potential. This second phase of depolarization is MK-801 sensitive and is therefore considered to be CSD (Gniel and Martin 2010) rather than AD induced by energy substrate deprivation, which is not blocked by MK-801 alone (Martin 1999; Muller and Somjen 1998). Spontaneously arising depolarizations in the ischemic penumbra and after cortical trauma that are known as peri-infarct depolarizations (PIDs) are likely to be a mixture of CSD and AD since the number of such depolarizations falls dramatically in the presence of MK-801 (Mies et al. 1993).

The first episode of CSD shifted membrane potential by 29.1 ± 4.0 mV to −2.0 ± 3.4 mV, whereas a second CSD—initiated 15 min later—shifted membrane potential by only 9.2 ± 3.7 mV, bringing it to −22.5 ± 3.2 mV (averaged values of \(n = 6\) cells for each CSD induction; representative trace presented in Fig. 1A). Both the membrane potential and the [Ca\(^{2+}\)]\text{i} returned to resting levels between episodes of CSD in each of these six neurons. The smaller change in membrane potential was accompanied by an increase in [Ca\(^{2+}\)]\text{i} to 0.6 ± 0.4 \(\mu\)M, compared with the increase to 1.8 ± 0.6 \(\mu\)M during the first CSD. With a third episode of CSD, membrane potential shifted by just 6.0 ± 2.3 mV to −28.3 ± 3.8 mV and [Ca\(^{2+}\)]\text{i} increased to 0.3 ± 0.1 \(\mu\)M. The attenuations of the membrane depolarization and peak [Ca\(^{2+}\)]\text{i} were highly significant (\(P < 0.0001\) and \(P = 0.0002\), respectively, 1-way ANOVA) (Fig. 1B). In these same experiments, the delay between administering the CSD-inducing ACSF and recording the negative shift in the DC potential increased significantly with successive episodes of CSD (1st episode: 148 ± 13 s, 2nd episode: 184 ± 22 s, 3rd episode: 207 ± 11 s; \(P < 0.0005\), 1-way ANOVA). This can be seen in the trace in Fig. 1A, where the delay is apparent as an increasing plateau immediately prior to the sudden shift in membrane potential that is CSD.

To determine whether the smaller increase in [Ca\(^{2+}\)]\text{i}, associated with the second and third episodes of CSD could be accounted for by the diminished depolarization, a voltage-clamp protocol, designed to model the changes in membrane potential that occurred during CSD, was used (see MATERIALS AND METHODS). Each cell (\(n = 6\)) was subjected to three families of voltage steps, delivered at 15-min intervals. The first family of voltage steps peaked at about −15 mV (compared with a CSD-induced peak of 2.1 mV) and was associated with an increase in [Ca\(^{2+}\)]\text{i} to 1.6 ± 0.2 \(\mu\)M (cf. 1.8 \(\mu\)M in CSD; \(P = 0.2\), unpaired Student’s t-test). The second family of voltage steps had a peak depolarization of −24.6 mV (cf. −22.5 mV in CSD) and induced a [Ca\(^{2+}\)]\text{i} increase to 0.9 ± 0.4 \(\mu\)M (cf. 0.6 \(\mu\)M in CSD; \(P = 0.22\), unpaired Student’s t-test). The third family of voltage steps, initiated 15 min after the second, peaked at −30.4 mV (cf. −28.3 mV) and was associated with a peak [Ca\(^{2+}\)]\text{i} of 0.6 ± 0.2 \(\mu\)M (cf. 0.3 \(\mu\)M; \(P = 0.03\), unpaired Student’s t-test; not shown). Thus the smaller depo-
larization associated with repeated episodes of CSD largely accounts for the smaller peak in \([\text{Ca}^{2+}]_i\).

To ascertain whether the diminishing response to repeated episodes of CSD was an artifact arising from whole cell recording, a “post-CSD patch technique” was employed. This involved inducing an initial episode of CSD with only the DC electrode in place. Toward the end of the inter-CSD interval a neuron was patched, and a second episode of CSD was then initiated 15 min after the first. Under these conditions the membrane potential shift was 10.2 ± 4.6 mV, bringing membrane potential to 21.0 ± 3.8 mV (n = 5) (Fig. 2), not significantly different from the amplitude of the depolarization when the patch pipette was in place for both the first and second episodes of CSD (P = 0.7, unpaired Student’s t-test).

The change in \([\text{Ca}^{2+}]_i\) could not be determined in these experiments as the pixel intensity of fura-4F was too low because the brief interval between patching a neuron and inducing CSD did not allow complete dialysis of the neuron with the dye. These results indicate that layer V pyramidal neurons show an attenuated response to a second episode of CSD initiated 15 min after the first, irrespective of the presence or absence of the patch electrode.

When the inter-CSD interval was increased to 30 min, the response to a second episode of CSD showed only minor attenuation, with the membrane depolarizing by 22.0 ± 2.7 mV to −7.3 ± 5.6 mV (n = 6; representative trace provided in Fig. 3A). A third episode of CSD elicited a further 30 min later shifted the membrane potential by 18.9 ± 6.6 mV to −10.4 ± 6.7 mV. Taken across all three CSD episodes the attenuation of CSD was significantly less than that observed across the three

Fig. 1. Response to repeated episodes of cortical spreading depression (CSD) at 15-min intervals in layer V cortical neurons. A: representative trace. CSD was induced with three 2-min applications of CSD-initiating ACSF (black bars, x-axis). The presence of 3 distinct DC shifts recorded in layer II/III (top) confirms that induction of CSD was successful, yet successively smaller shifts in membrane potential (middle) and intracellular \([\text{Ca}^{2+}]_i\); gray trace) occurred in the layer V neuron. B: averaged response for 6 neurons for all groups (mean ± SD). \(V_m\), membrane potential.

Fig. 2. The diminished membrane depolarization in response to a second episode of CSD 15 min after the first is not an artifact of patching: representative trace. During the first induction of CSD (black bar at 1–3 min) only the DC potential in layer II/III was recorded. At 15 min a layer V neuron was patched and a second episode of CSD was initiated. The membrane depolarized to a similar extent as it did when the patch electrode was present throughout both episodes of CSD (gray trace).
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CSD episodes at 15-min intervals ($P = 0.01$, repeated-measures analysis; Fig. 3B).

Over the course of the long experiments with a 30-min interval between CSD inductions the fluorescence intensity of the cell steadily declined to pixel intensities that were unreliable for determination of $[\text{Ca}^{2+}]_i$. To circumvent this problem the post-CSD patch technique was again employed. After the first CSD was initiated a 10-min interval followed to allow recovery of membrane potential before a layer V neuron was patched. This provided between 15 and 20 min for the fura-4F to equilibrate inside the cell before a second episode of CSD was initiated 30 min after the first, ensuring sufficiently high and stable pixel intensities for determination of $[\text{Ca}^{2+}]_i$ (not shown).

In these experiments the amplitude of the membrane potential shift in response to a second episode of CSD 30 min after the first was not significantly different from that measured in a second CSD when the patch electrode was in place for both the first and second CSDs (depolarization of $24.1 \pm 1.5$ mV, to $-5.4 \pm 1.9$ mV; $n = 5$; $P = 0.30$, unpaired Student’s $t$-test) and was therefore also significantly attenuated compared with a single CSD ($P = 0.002$, unpaired Student’s $t$-test). During the second episode of CSD the $[\text{Ca}^{2+}]_i$ increased to $1.6 \pm 0.22$ µM ($n = 5$), which was slightly, but not significantly, smaller than the peak increase in $[\text{Ca}^{2+}]_i$ resulting from a single CSD ($1.8 \pm 0.7$ µM; $P = 0.2$, unpaired Student’s $t$-test; data not shown). The delay between application of the CSD-initiating ACSF and the negative shift in the DC electrode remained present when the inter-CSD interval was extended to 30 min. Although this did not quite reach significance ($P = 0.06$, 1-way ANOVA), in the absence of one outlier value the result is clear ($P = 0.001$, 1-way ANOVA; data not shown).

In summary, these data indicate that in layer V pyramidal neurons a single episode of CSD “preconditions” against further CSD. The preconditioning lasts $\sim 30$ min and is expressed as a diminished change in membrane potential and peak $[\text{Ca}^{2+}]_i$ in repeated CSD, as well as an increased delay in the onset of subsequent episodes of CSD. The smaller change in peak $[\text{Ca}^{2+}]_i$ is largely due to the reduced membrane depolarization. The preconditioning is not an artifact of whole cell recording.

CSD-induced preconditioning of a subsequent exposure to OGD. To determine whether the CSD-induced preconditioning observed in layer V neurons could also confer resistance against OGD, a single CSD was initiated and 7 min later the perfusate was changed to OGD ACSF. This time interval was sufficient to allow neurons to fully repolarize from CSD before being subjected to in vitro ischemia. Two parameters were assessed to determine whether CSD was conferring a neuroprotective effect: 1) the increase in $[\text{Ca}^{2+}]_i$ occurring with the OGD-induced AD and 2) the duration of OGD application required to induce AD. Results were compared with same-day controls (different slices) in which AD was induced without prior CSD induction.

The peak increase in $[\text{Ca}^{2+}]_i$ resulting from AD following prior induction of a single episode of CSD was $2.8 \pm 0.8$ µM ($n = 6$). This was not significantly different from the increase to $3.2 \pm 1.0$ µM ($n = 4$) seen in same-day control cells.
exposed to OGD only ($P = 0.57$, unpaired Student’s $t$-test). As a more complete indicator of the Ca$^{2+}$ load, the integral of the [Ca$^{2+}$]$_i$ was calculated over a 6-min period from the onset of the rapid phase of depolarization. The integral in those cells previously exposed to a single episode of CSD was $13.2 \pm 6.3$ µm·min ($n = 6$), similar to the integral of $11.6 \pm 8.8$ µm·min ($n = 4$) in the relevant control cells exposed to OGD only ($P = 0.74$, unpaired Student’s $t$-test).

However, the time taken for AD to occur in response to OGD ACSF was significantly increased to $18.6 \pm 1.8$ min, compared with $11.7 \pm 2.0$ min for cells not previously exposed to CSD ($P < 0.0002$, unpaired Student’s $t$-test).

**CSD in conditions of metabolic stress.** In a further series of experiments we investigated the effect of CSD on neurons in the earliest phase of energy stress at the time of CSD induction, in an attempt to mimic CSD in the ischemic penumbra. The entire slice was subjected to metabolic compromise by OGD prior to CSD induction. The small hyperpolarization that reliably precedes AD was used as the indicator that metabolic compromise had begun to occur, and CSD was induced as soon as it was observed. On average OGD exposure lasted for $8.3 \pm 2.0$ min ($n = 5$), and at the time of CSD induction [Ca$^{2+}$]$_i$ was unchanged from pre-OGD values. The rapid phase of the CSD-induced depolarization had an amplitude of $26.4 \pm 2.0$ mV, bringing membrane potential to $-4.5 \pm 2.4$ mV, not significantly different from the amplitude of a single CSD-induced depolarization without prior OGD exposure ($n = 6$; $P = 0.21$, unpaired Student’s $t$-test). The peak increase in [Ca$^{2+}$]$_i$ associated with this depolarization was $1.6 \pm 0.2$ µM, also not significantly different from that induced by CSD in control conditions ($P = 0.6$, unpaired Student’s $t$-test). The calcium integrals were also similar between the two groups ($1.6 \pm 0.4$ µm·min, cf. $1.7 \pm 0.9$ µm·min in control conditions).

In this series of experiments, when a second episode of CSD was initiated 15 min after the first a significantly greater attenuation of the membrane depolarization occurred: $4.7 \pm 2.4$ mV compared with $9.2 \pm 3.7$ mV for a second episode of CSD in control conditions ($n = 5$; $P = 0.03$, unpaired Student’s $t$-test; representative trace in Fig. 4). The peak [Ca$^{2+}$]$_i$ associated with the depolarization was $0.2 \pm 0.1$ µM, significantly smaller than the peak of $0.6 \pm 0.4$ µM recorded in control conditions ($P = 0.03$, unpaired Student’s $t$-test). The integral of the calcium increase associated with the second episode of CSD was $0.3 \pm 0.1$ µm·min, not significantly different from the integral of $0.5 \pm 0.5$ µm·min measured in control conditions ($P = 0.42$, Student’s unpaired $t$-test). In fact, the response of OGD-exposed cells to a second episode of CSD was very similar to the response of control neurons to a third episode of CSD (representative trace in Fig. 4).

After return of [Ca$^{2+}$]$_i$ to stable baseline levels and repolarization of the membrane (complete in 2/5 neurons and within 5–10 mV in the remainder) after the second episode of CSD, OGD-exposed slices were returned to the OGD solution to induce AD. This took an average of $16.2 \pm 2.1$ min to occur ($n = 5$), significantly longer than the $12.9 \pm 2.4$ min required to elicit AD in neurons that had not been previously challenged with CSD ($n = 12$; $P = 0.02$, unpaired Student’s $t$-test). The AD-associated peak increase in [Ca$^{2+}$]$_i$ was $1.8 \pm 0.6$ µM, which is also significantly smaller than the AD-associated increase in the relevant control neurons ($3.8 \pm 0.8$ µM; $P = 0.0002$, unpaired Student’s $t$-test). Furthermore, the calcium integral was markedly reduced to $5.4 \pm 3.1$ µm·min compared with $17.9 \pm 4.6$ µm·min in the relevant control neurons ($P < 0.0001$, unpaired Student’s $t$-test).

Taken together, these results suggest that prior exposure to either CSD or OGD preconditions neurons and provides a degree of protection against subsequent exposure to OGD.

**CSD in layer II/III.** In all experiments described above, the extracellular DC recording electrode was placed in layer II/III of the cortex. Surprisingly, we noted that although the amplitude of the depolarization of layer V neurons diminished with repeated episodes of CSD at 15-min intervals, the amplitude of the DC shift was not significantly different between the first and second episodes of CSD (1st 4.61 mV, 2nd 4.65 mV; $P = 0.43$, paired Student’s $t$-test; $n = 22$). Therefore, in further experiments the DC electrode was placed in layer V of the cortex. In this position the DC electrode showed a significant decrease in the amplitude of the shift recorded during the first

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**Fig. 4.** Responses to repeated episodes of CSD after brief oxygen-glucose deprivation (OGD) in layer V neurons: representative trace. Slices were exposed to OGD (dashed line, x-axis) until a 2-mV hyperpolarization of the membrane potential was observed (compared with baseline before OGD was initiated; arrow). The perfusate was then immediately switched to the CSD-initiating ACSF (black bar at 9–11 min) and exposed a second time 15 min later (black bar at 24–26 min).
and second episodes of CSD (1st 3.99 mV, 2nd 2.39 mV; \( P = 0.02 \), paired Student’s \( t \)-test; \( n = 11 \)).

Because dislodgement of the patch pipette from layer II/III neurons persistently occurred soon after the CSD depolarization, we used the post-CSD patch technique first to assess that these neurons could recover fully from CSD and then to study the effects of repeated episodes of CSD on them. We found that resting membrane potential and input resistance were similar in layer II/III neurons previously subjected to CSD (\( -77.1 \pm 3.6 \) mV and \( 116 \pm 32 \) \( \Omega \), respectively; \( n = 7 \)) and those layer II/III neurons not previously subjected to CSD (\( -76.6 \pm 2.3 \) mV and \( 113 \pm 35 \) \( \Omega \), respectively; \( n = 8 \)).

Statistical comparison of the resting \([\text{Ca}^{2+}]_i\) was not made, because of the inaccuracy of using a low-affinity indicator such as fura-4F for \([\text{Ca}^{2+}]_i\) concentrations below 100 nM (see MATERIALS AND METHODS). However, the combination of the measures of resting membrane potential and input resistance suggests that layer II/III pyramidal neurons fully recovered after a single episode of CSD.

Using the post-CSD patch technique, we found, in stark contrast with the results for layer V neurons, that layer II/III neurons fully depolarized in response to a second episode of CSD initiated 15 min after the first (Fig. 5). The amplitude of the membrane potential shift was \( 27.8 \pm 0.8 \) mV, and membrane potential reached \( -1.1 \pm 0.8 \) mV, virtually identical to the value of \( -1.3 \pm 1.2 \) mV reached with a first episode of CSD (\( P = 0.7 \), unpaired Student’s \( t \)-test). Initiation of a third episode of CSD 15 min after the second was equally effective at depolarizing the membrane, with a shift of \( 24.2 \pm 6.3 \) mV bringing membrane potential to \( -1.2 \pm 1.9 \) mV (data not shown).

These data are consistent with the data recorded by the extracellular DC electrode in layer II/III of the cortex and demonstrate that the preconditioning induced by CSD in layer V is lamina specific and not present in layer II/III.

Adenosine, acting at the A1 receptor, is mediating CSD-induced preconditioning. Initially, we tested the hypothesis that the CSD-induced preconditioning involved a \([\text{Ca}^{2+}]_i\)-dependent signaling cascade by chelating neuronal intracellular \([\text{Ca}^{2+}]_i\) with 20 mM BAPTA in the patch pipette. The \([\text{Ca}^{2+}]_i\) chelator did not completely suppress a rise in \([\text{Ca}^{2+}]_i\), with a peak increase to \( 0.3 \pm 0.3 \) \( \mu \)M recorded, compared with the increase to \( 1.8 \pm 0.7 \) \( \mu \)M seen in control conditions (\( P < 0.0001 \), unpaired Student’s \( t \)-test). Nevertheless, a second episode of CSD shifted the membrane potential by just \( 8.9 \pm 5.6 \) mV, similar to the \( 9.2 \pm 2.0 \) mV shift recorded in control conditions.

These results suggest that CSD-induced preconditioning is probably not mediated by a \([\text{Ca}^{2+}]_i\)-dependent intracellular signaling cascade. As a result, an extracellular mechanism that could account for the preconditioning was sought. Because of evidence suggesting that the adenosine A1 receptor mediates IPC in the heart, and because endogenous adenosine has been demonstrated to exhibit an inhibitory influence on the appearance of spreading depression (SD) in rat hippocampus (Kaku et al. 1994), the A1 receptor antagonist 8-CPT was assessed for its ability to block CSD-induced preconditioning in layer V pyramidal neurons. In these experiments the perfusate was changed to ACSF containing 1 \( \mu \)M 8-CPT immediately after the 2-min application of CSD-inducing ACSF; that is, 8-CPT was present during the entire 15-min inter-CSD intervals but not prior to induction of the first episode of CSD. The presence of 8-CPT in the inter-CSD interval did not appear to alter the resting \([\text{Ca}^{2+}]_i\), although very small changes would not be detected by the \([\text{Ca}^{2+}]_i\)-sensitive dye used.

In the presence of 1 \( \mu \)M 8-CPT a second episode of CSD was more effective than in control conditions at depolarizing the neuron, with membrane potential shifting by \( 16.4 \pm 4.6 \) mV (\( n = 6 \)) compared with \( 9.2 \pm 3.7 \) mV in control conditions. The peak increase in \([\text{Ca}^{2+}]_i\), associated with the second episode of CSD in the presence of 1 \( \mu \)M 8-CPT was \( 1.5 \pm 0.5 \) \( \mu \)M (cf. \( 0.6 \pm 0.4 \) \( \mu \)M in control conditions). The integral of the calcium increase in the presence of 1 \( \mu \)M 8-CPT was \( 1.2 \pm 0.5 \) \( \mu \)M·min (cf. \( 0.5 \pm 0.5 \) \( \mu \)M·min in control conditions). A third episode of CSD shifted the membrane potential by \( 17.7 \pm 3.8 \) mV to \( -11.3 \pm 6.6 \) mV and increased the \([\text{Ca}^{2+}]_i\) to \( 1.0 \pm 0.6 \) \( \mu \)M (cf. membrane potential shift of \( 6.0 \pm 2.3 \) mV and \([\text{Ca}^{2+}]_i\) peak of \( 0.3 \pm 0.1 \) \( \mu \)M in control conditions). The integral of the calcium increase in the presence of 1 \( \mu \)M 8-CPT was \( 1.4 \pm 0.6 \) \( \mu \)M·min (cf. \( 0.4 \pm 0.4 \) \( \mu \)M·min in control conditions).

Increasing the concentration of 8-CPT to 3 \( \mu \)M further increased the amplitude of the depolarization observed with the second and third episodes of CSD. A second episode of CSD in these conditions resulted in a membrane potential shift of 25.9 \( \pm 3.1 \) mV. The accompanying increase in \([\text{Ca}^{2+}]_i\), peaked at \( 1.7 \pm 0.2 \) \( \mu \)M. A third episode of CSD depolarized the membrane by \( 24.8 \pm 2.6 \) mV. The change in \([\text{Ca}^{2+}]_i\) during the third episode of CSD could not be quantified because of low fluorescence intensity. The failure of three of the six cells to fully repolarize after CSD in the presence of 3 \( \mu \)M 8-CPT (in control conditions and 1 \( \mu \)M 8-CPT all cells fully or nearly fully repolarized) deterred investigation of the effects of a higher concentration of 8-CPT.

Figure 6A illustrates the amplitude of the membrane depolarizations seen in response to three consecutive episodes of CSD across all three treatment groups (control, 1 \( \mu \)M 8-CPT, and 3 \( \mu \)M 8-CPT). Analysis of the data for a treatment vs. time effect demonstrated a significant interaction (\( P = 0.003 \), repeated-measures analysis). Because it was not possible to reliably measure the changes in \([\text{Ca}^{2+}]_i\), for a third episode of CSD in the 3 \( \mu \)M 8-CPT group, the effect of 8-CPT on changes \([\text{Ca}^{2+}]_i\) could not be analyzed in the same way. However, the
that NMDA receptors were partially blocked during the first episode of CSD. Thereafter, the slice was superfused with the control ACSF, allowing the conversion of AMP to adenosine by ecto-5'-nucleotidase. Therefore, the role of extracellular adenosine in CSD-induce pre-conditioning was more directly investigated by using α,β-methyleneadenosine 5'-diphosphate (me-ADP) to inhibit the conversion of AMP to adenosine by ecto-5'-nucleotidase.

Fig. 7. Antagonism of the adenosine A1 receptor exacerbated the increasing delay to CSD induction. With successive inductions of CSD, the delay between applying the high-K+ solution and recording the shift in the DC potential increased in a sequential manner. This lengthening delay was enhanced by antagonism of the adenosine A1 receptor with 8-CPT (mean ± SD; P = 0.002, repeated-measures analysis; n = 6).
from those associated with the first episode of CSD in these cells \((P = 0.4, \text{paired Student’s } t\text{-test})\) but are significantly greater than values measured during a second episode of CSD in control conditions \((P = 0.0002 \text{ and } P = 0.005, \text{respectively, unpaired Student’s } t\text{-test})\). In response to a third episode of CSD, neurons depolarized by \(27.0 \pm 2.8 \text{ mV}\) (Fig. 8). Analysis of the depolarizations induced by the three successive episodes of CSD in neurons exposed to me-ADP demonstrated that there was no significant attenuation of the amplitude \((P = 0.53, 1\text{-way ANOVA})\), indicating that inhibition of ecto-5’-nucleotidase by me-ADP had blocked the induction of preconditioning in these five cells.

In the sixth cell exposed to me-ADP, the amplitude of the depolarization arising from the second induction of CSD was \(9.1 \text{ mV}\), virtually identical to the average amplitude of the depolarization seen with a second episode of CSD in control conditions \((9.2 \pm 3.7 \text{ mV})\). Clearly, inhibition of extracellular adenosine formation had no effect on preconditioning in this neuron.

Inhibition of ENTs with DIPY produced variable effects on preconditioning. In a further set of experiments transport of adenosine through the ENTs was blocked with DIPY, a blocker of both ENT1 and ENT2 (Thorn and Jarvis 1996), or with a combination of DIPY and the potent ENT1 subtype inhibitor NBTI (Thorn and Jarvis 1996). The experimental protocol was slightly modified from that when APV and me-ADP were used insofar as the ENT inhibitor(s) was present for a further 3 min after initiation of CSD. Because DIPY is itself a fluorescent molecule, changes in \([\text{Ca}^{2+}]_i\) could not be monitored in these experiments.

Neither DIPY nor DIPY and NBTI affected resting membrane potential. However, with DIPY treatment the first CSD induced a shift in membrane potential shift of \(27.0 \pm 1.3 \text{ mV}\) \((n = 8)\), significantly smaller than the shift of \(29.1 \pm 4.0 \text{ mV}\) recorded in control conditions \((P = 0.005, \text{unpaired Student’s } t\text{-test}; n = 6)\). In response to the second induction of CSD,

Fig. 9. The presence of equilibrative nucleoside transporter (ENT) inhibitors produced heterogeneous effects on preconditioning. A: during exposure to dipyridamole (DIPY), responses to the first episode of CSD were homogeneous. The response to a second episode of CSD demonstrated that in 6 neurons preconditioning was largely abolished; however, in 2 cells the attenuation expected from preconditioning was observed. After a third induction of CSD, 2 neurons showed a near-full response while 4 neurons showed an attenuated response. B: DIPY + 6-[(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (NBTI) resulted in a variable response to a first episode of CSD, with 2 neurons showing a greatly reduced amplitude, suggesting a buildup of extracellular adenosine around these cells as a result of the more robust blocking of ENTs. The response to both the second and third episodes of CSD varied greatly.
ive of the eight cells depolarized to a slightly smaller extent than during the initial episode of CSD (Fig. 9A). The amplitude of the mean depolarization of these five cells was \(25.2 \pm 1.7 \text{ mV}\), which was a significant decrease compared with the first episode of CSD \((P = 0.02, \text{paired Student’s } t\text{-test})\) but much greater than the amplitude of the depolarization induced by a second episode of CSD in slices that had not been exposed to DIPY \((9.2 \pm 3.7 \text{ mV}; P < 0.0001, \text{unpaired Student’s } t\text{-test})\). The second CSD of the remaining three of the eight cells exposed to DIPY displayed virtually no attenuation of the preconditioning (Fig. 9B). The mean amplitude of the depo-
larization associated with a second episode of CSD in these neurons was 10.0 ± 1.6 mV, which is not significantly different from the mean amplitude recorded in response to a second episode of CSD in control conditions.

A third application of the CSD-initiating ACSF induced CSD in six of eight slices. Of the six cells studied, two showed almost full depolarization, with a shift in membrane potential of 25.0 ± 0.4 mV, whereas the other four neurons only depolarized by 6.2 ± 2.3 mV, similar to the amplitude of the depolarization seen in control cells during a third episode of CSD (6.0 ± 2.3 mV; \( P = 0.9 \), unpaired Student’s \( t \)-test).

Interestingly, after washout of DIPY the amplitude of the second DC shift significantly increased compared with the amplitude recorded during the first CSD in the presence of DIPY (6.7 ± 0.9 mV compared with 5.4 ± 0.8 mV; \( P = 0.001 \), paired Student’s \( t \)-test). Results with combined use of DIPY and NBTI were more heterogeneous than those with DIPY alone. Even in response to the first episode of CSD, 2 of 10 cells showed a markedly attenuated CSD (Fig. 9B). This suggests that in these slices there had been a major buildup of adenosine extracellularly before induction of CSD. A second CSD 15 min later produced a nearly full depolarization in three cells (mean = 27.6 ± 0.3 mV), a partial depolarization in four cells (mean = 18.1 ± 1.4 mV), and a small depolarization in the remaining three cells (mean = 8.0 ± 4.6 mV) comparable with the full preconditioning seen in control cells (Fig. 9B). A third episode of CSD was only induced in four cells, with two showing a nearly full depolarization and two a small depolarization comparable with the preconditioning of controls (Fig. 9B).

Again, a significant increase in the amplitude of the DC shift occurred after washout of DIPY + NBTI (6.3 ± 0.8 mV compared with 5.4 ± 0.8 mV; \( P < 0.01 \), paired Student’s \( t \)-test). Taken together, these data support an important role for ENTs in the preconditioning of CSD by CSD.

### Discussion

This report provides the first analysis of the effect of repeated episodes of CSD on the membrane potential and \([\text{Ca}^{2+}]_i\) of individual layer V and layer II/III pyramidal neurons. In layer V neurons a first episode of CSD protected neurons against successive episodes of CSD initiated at 15-min intervals, as demonstrated by the reduction in both the amplitude of the depolarization and the \([\text{Ca}^{2+}]_i\) load. In contrast, we found no such preconditioning in layer II/III pyramidal neurons; that is, with repeated episodes of CSD a full response occurred. The preconditioning effect of CSD in layer V pyramids was short-lasting, with a near-full response being recorded when the inter-CSD interval was extended to 30 min. The duration of the protective effect is shorter than that of classical preconditioning in the heart, which manifests itself immediately and lasts 2–3 h (Haushenloy and Yellon 2012). However, we believe preconditioning is the most appropriate term to describe the attenuation of the response.

We did not measure changes in the extracellular adenosine concentration in this study, but that elevation of extracellular adenosine occurs in CSD can be inferred from a variety of studies. First, propagation of CSD requires activation of NMDA receptors (Anderson and Andrew 2002; Lauritzen and Hansen 1992; Marrannes et al. 1988; Neillgard and Wieloch 1992; Peters et al. 2003; Vilagi et al. 2001), and activation of cortical NMDA receptors has been shown to increase the concentration of adenosine extracellularly (Craig and White 1993; Hoehn et al. 1990). Second, in CSD ATP is released extracellularly (Schock et al. 2007), and this will be broken down to adenosine by ecto-ATPases. Third, in vivo CSD is thought to be initiated, at least in part, by elevated extracellular \(K^+\) (Vyskocil et al. 1972), and \(K^+\)-induced depolarization also increases the extracellular adenosine concentration in various brain regions (Chen et al. 1992; Hoehn and White 1990; Van Wylen et al. 1986) including cortex (Hollins and Stone 1980; Pazzagli et al. 1994).

The CSD-induced preconditioning we report was reduced by 1 \(\mu\)M 8-CPT and largely blocked by 3 \(\mu\)M 8-CPT. This indicates \(A_1\) receptor involvement, as 8-CPT is considered to be a selective \(A_1\) receptor antagonist (Fredholm et al. 2001). Should 8-CPT at the higher concentration be acting nonselectively, then its most likely target is the \(A_{2A}\) receptor (Bruns et al. 1986). Given that expression of the \(A_{2A}\) receptor is very weak in the cortex (Dixon et al. 1996), then even at the higher concentration the effects of 8-CPT will be dominantly at \(A_1\) receptors.

The source of the adenosine arising from CSD would appear to be predominantly extracellular as demonstrated by the failure to observe preconditioning in five of six cells when the hydrolysis of AMP to adenosine by ecto-5′-nucleotidase was blocked by me-ADP. This is consistent with a study that demonstrated that ATP is released into the extracellular space (ECS) with each successive wave of CSD (Schock et al. 2007). However, the lack of effect of me-ADP in one cell of the six studied raised the possibility that adenosine release (either by transporters or from vesicles) also occurs. When we blocked ENTs with DIPY, which at the concentration we used is likely to partially block ENT1 but fully block the more abundant ENT2 (Kiss et al. 2000) of the mouse brain (Thorn and Jarvis 1996), the first CSD was slightly attenuated. This is most likely as a result of adenosine accumulation causing inhibition of synaptic transmission (Dunwiddie and Diao 2000). Almost complete suppression of preconditioning occurred in six of eight neurons during a second CSD but in only two of six neurons in a third episode of CSD. It appears that in these particular cells block of the ENTs had completely removed the source of adenosine for preconditioning. However, as more CSDs occurred increasing evidence of preconditioning appeared, probably as a result of ATP release and its extracellular degradation to adenosine by ecto-5′-nucleotidase. Nevertheless, we cannot rule out the possibility that the preconditioning of CSD in the presence of DIPY is by an adenosine-independent mechanism.

When ENTs were blocked with a combination of DIPY and the potent ENT1 inhibitor NBTI a similar result was obtained, although notably there was evidence of more preconditioning rather than less. With the very first CSD two cells showed a much smaller depolarization than we had ever measured in many control experiments. These results suggest that in these experiments the fuller block of the ENTs led to a buildup of adenosine before CSD was induced. Apparently, the effect of basal production of extracellular adenosine by enzymatic degradation of AMP was no longer being counterbalanced by uptake via ENTs. In subsequent episodes of CSD increasing evidence of buildup of extracellular adenosine is indicated.
although as noted above an adenosine-independent mechanism may be at play.

**Laminar specificity of response.** In a number of other studies, and initially in this one, the DC recording electrode used to measure the CSD-associated DC shift was placed in layer II/III of the cortex (Anderson and Andrew 2002; Footit 1998; Peters et al. 2003). In one of these studies, the reproducibility of the DC response measured at ~15-min intervals led the authors to assume that the CSD response had fully resolved (Anderson and Andrew 2002). However, by monitoring the changes in membrane potential of individual neurons from different layers of the cortex, we have demonstrated that the extracellular potential shift recorded in layer II/III does not provide a picture of the changes that occur in other layers of the cortex in response to CSD. Indeed, by measuring the membrane potential in individual neurons in each of layers II/III and layer V we show that neurons in these layers respond quite differently to successive episodes of CSD.

The lamina specificity of the preconditioning response is consistent with immunohistochemical studies of receptor densities in the rat cortex. Although A₁ receptors are present in all cortical layers, the large pyramidal neurons of layer V have the highest density of any cortical layer (Ochishii et al. 1999; Rivkees et al. 1995). The density of receptors in layer III is moderate, while the pyramidal neurons from layer II were found to have particularly sparse labeling (Rivkees et al. 1995). However, other mechanisms such as reduced ATP release, reduced ecto-ATPase activity for conversion of ATP to adenosine, or less effective coupling between receptor and intracellular messengers may be responsible for the differential lamina response.

Interestingly, block of ecto-5′-nucleotidase had no effect on the amplitude of the DC potential recorded in layer II/III, whereas block of ENTs increased the DC amplitude. This suggests that the basal level of adenosine had fallen and this would only be possible if the ENTs were usually predominantly transporting adenosine out of the cells.

**Duration of preconditioning.** Two separate mechanisms could be responsible for the CSD-induced preconditioning being limited in duration to ~30 min. The first possibility is that the time course reflects the duration of time that the extracellular adenosine concentration is raised. When the adenosine in the intracellular compartment begins to decrease as adenosine is either phosphorylated by adenosine kinase to form AMP or converted to inosine by adenosine deaminase, the movement of adenosine through ENTs will reverse and remove adenosine from the ECS (Fredholm et al. 2005). Adenosine can also be converted to inosine extracellularly, providing another mechanism for the extracellular concentration to be reduced.

A second possibility is that desensitization or internalization of the A₁ receptors could underlie the short time frame of CSD-induced preconditioning. There is controversy in the literature regarding the time frame over which A₁ receptors desensitize, with some researchers asserting that several hours is required (Hettinger et al. 1998; Wetherington and Lambert 2002). However, in the rat hippocampus Coelho et al. (2006) found that after 60 min of hypoxia A₁ receptors desensitized and internalized within ~90 min. In the rat striatum, even more rapid desensitization has been reported, with application of A₁ agonists resulting in desensitization within 15–30 min (Abbracchio et al. 1992), a time course that would fit with the experimental data presented here. Parallel radioligand binding studies carried out by Abbracchio and colleagues showed no changes in the binding patterns over this time, suggesting that the receptors remain in the plasma membrane.

**Delayed CSD onset.** A further observation to arise from the repeated induction of CSD was the increasing delay between perfusion of the slice with the CSD-inducing ACSF and the onset of CSD. The A₁ receptor antagonist 8-CPT exacerbated the delay. A decreased rate of propagation of the waveform, as has been reported in the retina during repeated induction of SD (Brand et al. 1997; Gorelova and Bures 1983; Weimer and Hanke 2005), could be responsible, although the reason for the reduced rate of propagation is not known. Another possibility is the finding that synaptic release and extracellular accumulation of Zn²⁺ occurs in CSD, and this has recently been demonstrated to decrease the propagation rate of CSD in murine brain slices on a timescale consistent with our data (Aiba et al. 2012). Nevertheless, we cannot rule out NMDA receptor inhibition by some unidentified mechanism as the cause of the delay in onset of CSD that we report.

**CSD after brief metabolic compromise.** There is significant evidence indicating that CSD exacerbates damage arising from an ischemic event by applying further stress to the metabolically compromised neurons in the penumbra (Back et al. 1996; Busch et al. 1996; Takano et al. 1996).

To investigate the effect of CSD after brief metabolic compromise, the entire slice was briefly exposed to OGD prior to CSD induction. We found a changed response only to a second episode of CSD, where a greater attenuation of both membrane potential and the peak increase in [Ca²⁺], occurred than in a second CSD in normoxic conditions. Apparently OGD itself did not provide protection against a subsequent CSD, but the ability of one CSD to protect against the next was enhanced relative to control conditions.

Furthermore, in these neurons that had been exposed to brief metabolic compromise prior to the induction of CSD the increase in [Ca²⁺], associated with a subsequent AD was significantly reduced. This finding is consistent with a previous study that demonstrated that the Ca²⁺ influx in CA1 pyramidal neurons arising from a sustained ischemic insult was decreased in gerbils preconditioned with a brief ischemic insult (Shimazaki et al. 1998). Given the evidence outlined here that adenosine acting at the A₁ receptor mediates CSD-induced preconditioning, this suggests that either more adenosine is entering the ECS after energy deprivation or the downstream effects of A₁ receptor activation have been potentiated. As the extracellular concentration of adenosine has been shown to increase far more in response to hypoxia, anoxia, and ischemia than with K⁺–induced depolarization (Hagberg et al. 1987; Latini et al. 1999; Matsumoto et al. 1992) the former of these possibilities is the more likely. However, the response to an initial episode of CSD is not smaller than in control conditions, suggesting that there is a requisite lag time required for this process to be activated. Thus a downstream effect cannot be discounted.

**CSD protects against OGD.** Additionally, we found that a single episode of CSD protected against subsequent exposure to OGD. Although AD still occurred in these neurons, the length of time neurons could tolerate OGD before depolarization was increased by nearly 60%. This result is not without precedence: activation of the A₁ receptor with the potent agonists resulting in desensitization within 15–30 min (Abbracchio et al. 1992), a time course that would fit with the
agonist N\textsuperscript{6}-cyclopentyladenosine (CPA) has been demonstrated to inhibit hypoxia-induced depolarization in the somatosensory cortex of the rat (Nieber et al. 1999). However, in an investigation of the effect of anoxia on hippocampal slices Perez-Pinzon et al. (1996) found that neither adenosine nor adenosine analogs increased the time to AD onset. Given that our results are consistent with those of Nieber et al. (1999), who were also working in the cortex, this may reflect a difference between brain regions.

Neither the increase in [Ca\textsuperscript{2+}], or the calcium integral associated with the delayed AD was altered by the prior induction of CSD. This suggests that AD occurred once the preconditioning afforded by CSD had diminished below an effective threshold, and that all avenues for raising cytosolic Ca\textsuperscript{2+} were available. Nevertheless, there may have still been protection conferred through the prior induction of CSD, as reported by Bickler and Fahlman (2004). In this study a moderate increase in [Ca\textsuperscript{2+}], 30 min prior to OGD induction resulted in significantly less cell death in hippocampal CA1 and CA3 neurons, despite the fact that the increase in [Ca\textsuperscript{2+}] associated with the OGD exposure was unchanged. This was proposed to result from calcium-dependent upregulation of the Akt and MAP kinase pathways, which are implicated in the induction of apoptosis.

The data presented here may appear to be at odds with the body of evidence linking PIDs, and also CSDs specifically, to infarct expansion. However, that is not necessarily the case. The immediate preconditioning effect observed here lasted only 30 min. If the cessation of the preconditioning is being mediated by desensitization of A\textsubscript{1} receptors, this may allow the less abundant A\textsubscript{2A} receptors to be activated. In contrast to A\textsubscript{1} receptors, A\textsubscript{2A} receptors are believed to be detrimental after ischemia, as demonstrated by a number of studies that have shown antagonism of A\textsubscript{2A} receptors to be neuroprotective after in vivo ischemia (Chen et al. 1999; Gao and Phillis 1994; Melani et al. 2003; Monopoli et al. 1998; Von Lubitz et al. 1995). Therefore, a window may exist for adenosine, acting at A\textsubscript{2A} receptors, to adversely affect neurons after desensitization of A\textsubscript{1} receptors and prior to the expression of neuroprotective genes.

In summary, a single episode of CSD induced short-lasting preconditioning in layer V pyramidal neurons that was largely abolished 30 min after CSD induction. The preconditioning conferred protection against both subsequent CSD and OGD. The preconditioning afforded by CSD had lamina specific, being absent in layer II/III pyramidal neurons. Blockade of the adenosine A\textsubscript{1} receptor with the selective antagonist 8-CPT diminished the degree of preconditioning observed in a concentration-dependent manner, indicating that extracellular adenosine was mediating the effect. Blocking extracellular formation of adenosine by ecto-5'-nucleotidase with me-ADP abolished preconditioning in most, but not all, cells. Block of ENT1 and ENT2 with DIPY alone or in combination with NBtI also prevented preconditioning in some, but not all, cells. These data provide evidence that rapid preconditioning of one CSD by another is primarily mediated by adenosine.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: H.M.G. and R.L.M. conception and design of research; H.M.G. performed experiments; H.M.G. analyzed data; H.M.G. and R.L.M. interpreted results of experiments; H.M.G. prepared figures; H.M.G. drafted manuscript; H.M.G. and R.L.M. edited and revised manuscript; H.M.G. and R.L.M. approved final version of manuscript.

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CSD INDUCES LAMINA-SPECIFIC PRECONDITIONING


