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Neuronal activity regulates astrocytic Nrf2 signaling

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Nuclear factor erythroid 2-related factor 2 (Nrf2), the transcriptional master regulator of the stress-induced antioxidant response, plays a key role in neuronal resistance to oxidative stress and glutamate-induced excitotoxicity. Nrf2-mediated neuroprotection is primarily conferred by astrocytes both in vitro and in vivo, but little is known about physiologic signals that regulate neuronal and astrocytic Nrf2 signaling. Here, we report that activity of the Nrf2 pathway in the brain is fine-tuned through a regulatory loop between neurons and astrocytes: elevated neuronal activity leads to secretion of glutamate and other soluble factors, which activate the astrocytic Nrf2 pathway through a signaling cascade that involves group I metabotropic glutamate receptors and intracellular Ca^{2+}. Therefore, regulation of endogenous antioxidant signaling is one of the functions of the neuron–astrocyte tripartite synapse; by matching the astrocyte neuroprotective capacity to the degree of activity in adjacent neuronal synapses, this regulatory mechanism may limit the physiologic costs associated with Nrf2 activation.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine-zipper transcription factor, regulates both baseline and inducible expression of a battery of antioxidant and phase II detoxification enzymes (1). Under baseline conditions, Nrf2 is targeted for proteasomal degradation; stimuli that activate the Nrf2 pathway [oxidative stress, kinase activation, or treatment by small molecules, such as sulforaphane (SLF)] lead to Nrf2 translocation into the nucleus and an increase in the transcription of genes that contain the ARE (antioxidant response element) in the 5′ regulatory region (2).

Nrf2-null mice develop diffuse white matter injury without overt loss of neurons (3). However, when these animals are exposed to mitochondrial toxins or oxidative stressors, they show increased susceptibility to neurodegeneration (4, 5). In cortical cultures, Nrf2 signaling is critical for neuronal resistance to mitochondrial complex I inhibitors, excessive Ca^{2+} influx, and glutamate-induced excitotoxicity (6). Interestingly, Nrf2-mediated neuroprotection is primarily conferred by astrocytes both in vitro (7, 8) and in vivo, where selective overexpression of Nrf2 under an astrocyte-specific promoter leads to increased survival in mouse models of Parkinson disease (9) and amyotrophic lateral sclerosis (10). However, little is known about physiologic signals that regulate Nrf2 signaling in the CNS.

Increased synaptic activity protects neurons from apoptosis induced by staurosporine (11) or oxidative stress (12) through up-regulation of antiapoptotic genes (13) and activation of intrinsic antioxidant defenses through FOXO, C/EBPβ, and AP-1 signaling pathways (12). It is currently not known, however, whether increased synaptic activity can activate the Nrf2 pathway in either neurons or glia. Perisynaptic astrocytes, which together with pre- and postsynaptic neurons form a "tripartite synapse," regulate neuronal excitability and strength of synaptic transmission (14). Here, we show that neuron–astrocyte interactions also play a key role in the regulation of brain Nrf2 signaling.

Results

Neuronal Activity Potentiates Nrf2 Signaling in Mixed Hippocampal Cultures. To investigate whether Nrf2 signaling is regulated by neuronal activity, we used mixed and predominantly neuronal primary hippocampal cultures (Fig. 1*A*). Neuronal activity was induced by treatment with 50 mM K^+ (high K^+) artificial cerebrospinal fluid (ACSF), a commonly used depolarizing stimulus that triggers a burst of action potentials and global neurotransmitter release (15). High K^+ treatment increased the level of nuclear 84-kDa Nrf2 protein in mixed but not in neuronal or astrocytic cultures (Fig. 1*B*). In contrast, treatment with SLF (a direct activator of Nrf2 pathway) increased the nuclear Nrf2 protein in both mixed and astrocytic, but not in neuronal cultures (Fig. 1*B*). [Commercially available Nrf2 antibodies detect multiple protein bands, but only the 84-kDa band was increased by SLF treatment and attenuated by Nrf2 siRNA transfection in astrocyte cultures (Fig. S1*A*), indicating that it was specific for Nrf2. In all subsequent Western blotting experiments, SLF treatment was used as a positive control to unambiguously identify the specific Nrf2 band.]

Hippocampal cultures predominantly consist of excitatory glutamatergic neurons, but also contain a small number of GABAergic neurons that inhibit network activity (11). To investigate whether Nrf2 signaling can be activated through a local (synapse-restricted) neurotransmitter release, we treated cultures with $GABA_A$ receptor antagonist gabazine (Gab) and K^+ channel antagonist 4-aminopyridine (4-AP). Both Gab and 4-AP increase neuronal firing frequency and Ca^{2+} influx associated with activation of synaptic (but not extrasynaptic) NMDA receptors (NMDARs); combining the two drugs leads to a stronger response while preserving the high spatial specificity (11). Like high K^+, Gab/4-AP treatment increased nuclear Nrf2 protein level in mixed but not in neuronal or astrocytic cultures (Fig. 1*C*); weaker activation was seen when cultures were treated with either drug alone (Fig. S1*B*). Similar results were obtained with other $GABA_A$ receptor antagonists (bicuculline or picrotoxin) (Figs. S1 *C* and *D*). Interestingly, activation of Nrf2 signaling by Gab/4-AP required at least overnight treatment, whereas high K^+ was effective after as little as 2 h (Figs. S1 *E* and *F*).

To confirm that increase in the nuclear Nrf2 protein level results in activation of downstream Nrf2 signaling, we used quantitative RT-PCR (qRT-PCR) to measure mRNA levels of a subset of Nrf2-regulated genes. In mixed cultures, both high K^+ and Gab/4-AP treatments increased levels of Nqo1 and Gclc mRNAs,

Significance

Tripartite synapse, which consists of presynaptic neuron, postsynaptic neuron, and perisynaptic astrocyte, is the central site of intercellular communication in the brain. Here, we show that neuron–astrocyte signaling at the tripartite synapse also regulates activity of nuclear factor erythroid 2-related factor 2 (Nrf2), the transcriptional master regulator of the stress-induced cytoprotective response: through secretion of neurotransmitter glutamate and other soluble messengers, elevated neuronal activity leads to increased expression of Nrf2-regulated genes in neighboring astrocytes. By optimizing astrocyte neuroprotective capacity to the level of adjacent synaptic activity, this regulatory mechanism may contribute to the maintenance of brain health under physiological stress.

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The authors declare no conflict of interest.

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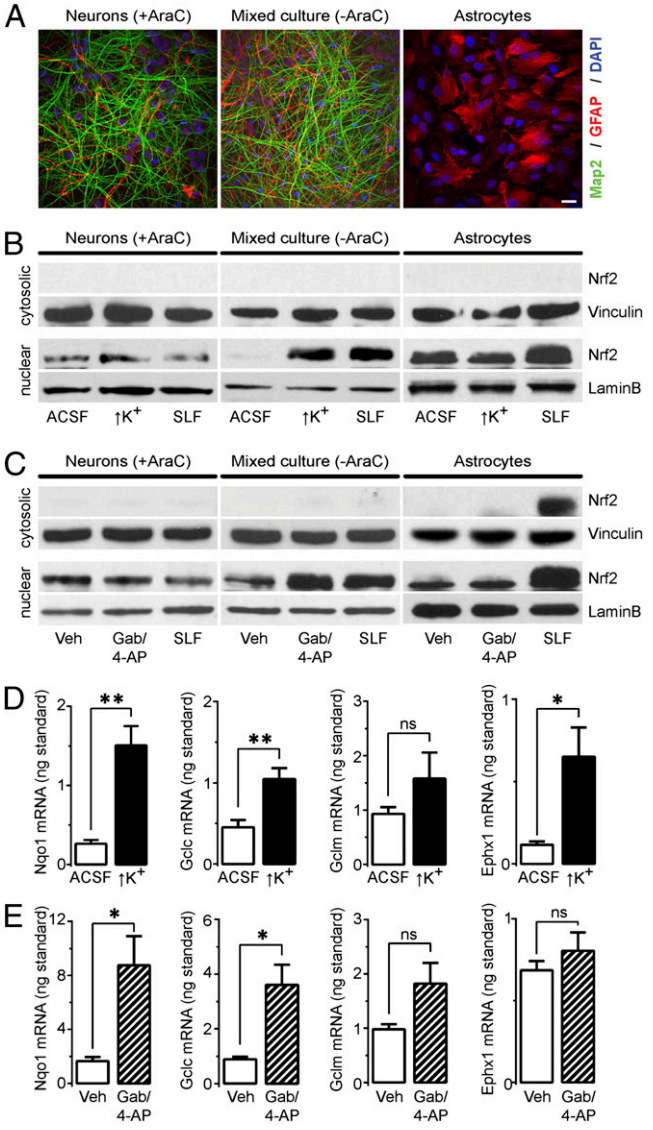

Fig. 1. High K^+ and Gab/4-AP treatments activate Nrf2 signaling in mixed hippocampal cultures. (*A*) Neuronal, mixed, and astrocytic cultures were immunostained for Map2 (neuronal marker, green) and GFAP (astrocytic marker, red); DAPI nuclear staining (blue) was used to visualize all cells. At DIV14, mixed cultures were composed of 49 ± 9% neurons, 17 ± 2% astrocytes, and 33 ± 11% other cells (presumably oligodendroglia and microglia); AraC-treated, neuron-enriched cultures consisted of 95 ± 2% neurons, 4 ± 2% astrocytes, and 1 ± 1% other cell types (mean ± SD, $n = 3$). In astrocytic cultures, no neurons were present and nearly all nuclei were associated with $GFAP^+$ cell bodies. Note that astrocytes cultured with neurons extend many long processes, but astrocytes cultured alone exhibit a flat, epithelioid morphology. (Scale bar, 50 μm.) (*B* and *C*) Neuronal, mixed, and astrocytic cultures were treated with control or 50 mM K^+ ($\uparrow K^+$) ACSF for 4 h (*B*), 0.1% DMSO (Veh), or 20 μM Gab + 2.5 mM 4-AP (Gab/4-AP) for 24 h (*C*), or 2.5 μM SLF for 16 h (*B* and *C*). Nuclear Nrf2 protein level (apparent molecular weight: 84 kDa) was increased by high K^+ and Gab/4-AP treatments only in mixed cultures. Cytosolic Nrf2 protein level was generally below the detection threshold; however, an increase in cytosolic Nrf2 was occasionally seen in SLF-treated astrocytic cultures (*C*). Representative of five or more similar experiments is shown. (*D* and *E*) Mixed cultures were treated with control or high K^+ ACSF for 4 h (*D*) or with 0.1% DMSO (Veh) or Gab/4-AP for 24 h (*E*). A statistically significant increase in Nqo1 and Gclc mRNA was seen with both treatments, but only high K^+ treatment led to increase of Ephx1 mRNA; the level of Gclm mRNA was not significantly changed after either treatment. Note that both baseline and induced levels for all mRNAs (except Gclm) were lower in ACSF than in the culture medium, which was used for Gab/4-AP treatment. $*p < 0.05$; $**p < 0.01$ (mean ± SEM, two-tailed *t* test, $n = 5$). ns, not significant.

but Ephx1 mRNA was increased only after high K^+ treatment (Figs. 1 *D* and *E*). In predominantly neuronal cultures, we measured a similar relative increase but smaller absolute levels for each mRNA (Fig. S2); given that neuronal cultures contain a small fraction of nonneuronal cells (Fig. 1*A*), these results suggest that neuronal firing increases Nrf2 activity in glia rather than neurons.

Neuronal Activity Activates Nrf2 Signaling in Astrocytes Through Soluble Factors. Mixed hippocampal cultures are primarily composed of neurons and astrocytes, but other glia are also present (Fig. 1*A*). To determine whether neurons and astrocytes are sufficient for Nrf2 activation by neuronal activity, we plated separately cultured astrocytes into cytosine arabinoside (AraC)-treated neuronal cultures (Fig. 2*A*). Treatment of these direct neuron–astrocyte cocultures with high K^+ resulted in nuclear Nrf2 protein increase comparable to that seen in mixed cultures; no increase was observed in neuronal cultures that were not seeded with astrocytes (Fig. 2*B*). A similar result was seen if Gab/4-AP treatment was used to induce synaptic activity (Fig. 2*C*). In these experiments, neurons were cultured with astrocytes for 6 d before treatment; however, the same results were seen when the coculture period was shortened to 1 d (Fig. S3).

To confirm that the Nrf2 pathway activation occurs in astrocytes rather than neurons (as suggested by results of qRT-PCR experiments), we used Gab/4-AP treatment to increase action potential frequency in brain slices from ARE-human placental alkaline phosphatase (hPAP) transgenic reporter mice (16) (Fig. S4). Both in control and Gab/4-AP–treated slices (Fig. 3 *A*–*C*), $hPAP^+$ cells displayed characteristic astrocyte morphology [a small cell body with highly branched fine processes (17)]; no $hPAP^+$ cells with neuronal morphology (17) were observed (Fig. 3*C*). The number of $hPAP^+$ astrocytes was significantly higher in Gab/4-AP–treated than in vehicle-treated slices and was comparable to the number of $hPAP^+$ astrocytes in SLF-treated slices;

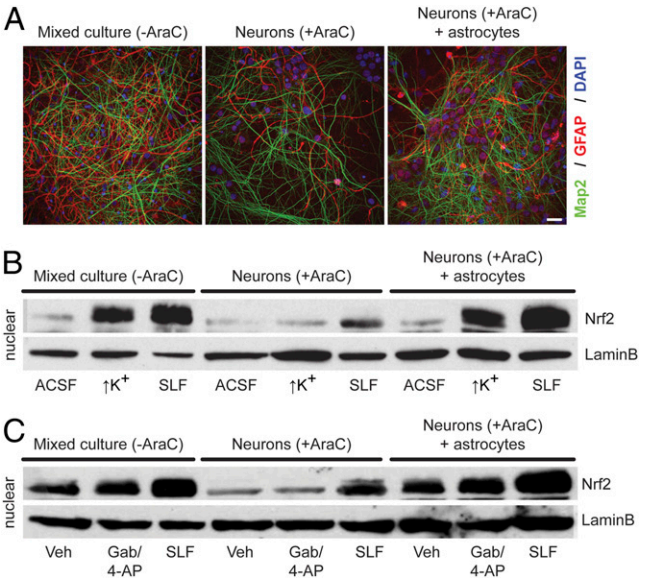

Fig. 2. Nrf2 pathway activation by high K^+ or Gab/4-AP requires neurons and astrocytes. Direct neuron–astrocyte cocultures were obtained by plating astrocytes into DIV10 neuronal cultures, with experiments performed 6 d after astrocyte plating. (*A*) Mixed cultures, neuronal cultures, and direct neuron–astrocyte cocultures were immunostained for Map2 and GFAP as in Fig. 1; DAPI nuclear staining (blue) was used to visualize all cells. A representative of three experiments is shown. (Scale bar, 50 μm.) (*B* and *C*) Culture treatments were performed as described in Fig. 1. Nuclear Nrf2 protein level was increased by high K^+ and Gab/4-AP treatments to a similar extent in mixed cultures and direct neuron–astrocyte cocultures; a representative of three or more similar experiments is shown.

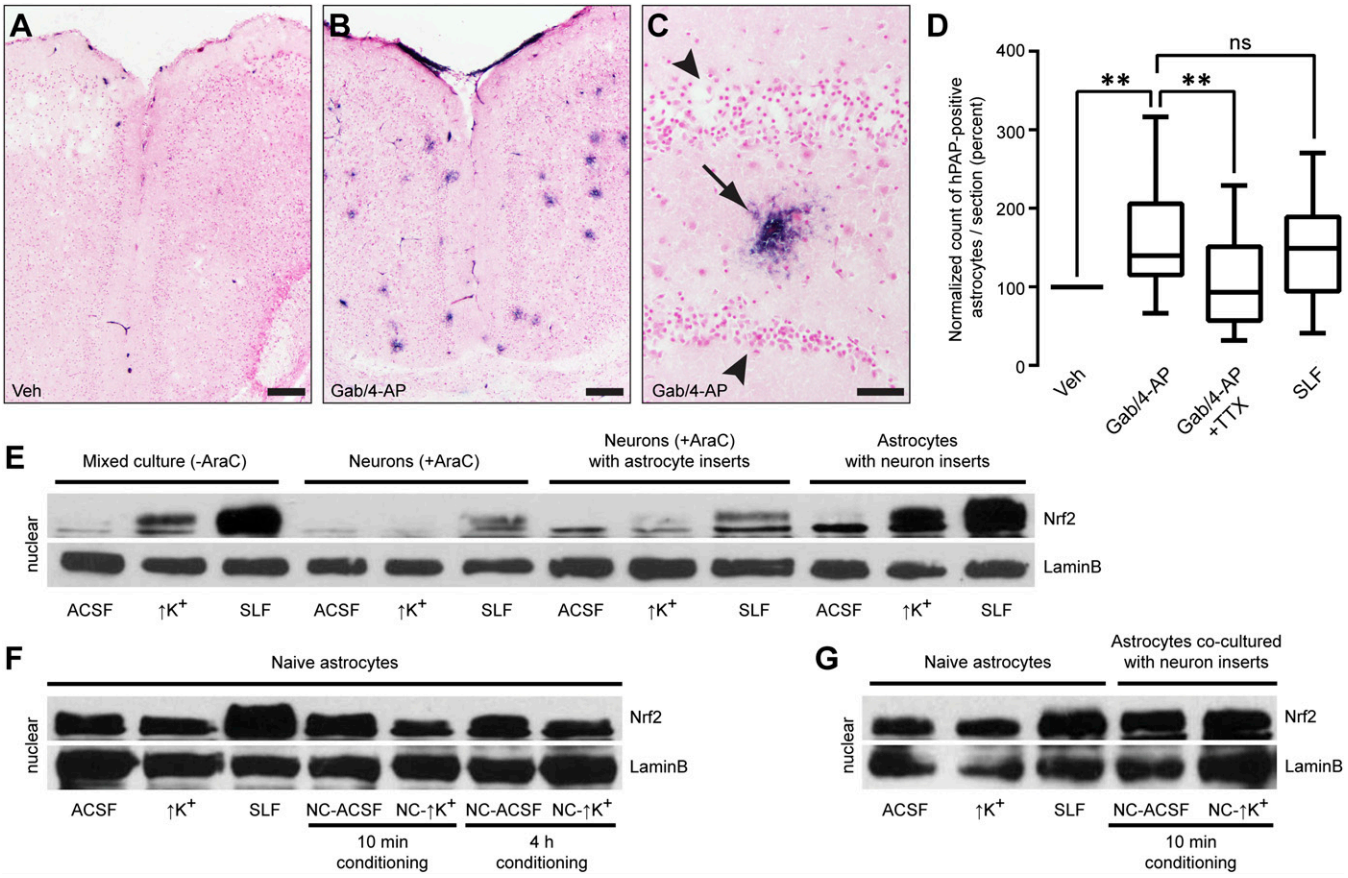

Fig. 3. Neuronal activity induces Nrf2 signaling in astrocytes through a diffusible messenger. (*A*–*C*) Following Gab/4-AP treatment, hPAP$^+$ cells (arrow in *C*) showed astrocytic morphology (*A* and *B*, frontal cortex; *C*, hippocampus); neurons were not hPAP$^+$ (arrowheads in *C* mark the two blades of the dentate gyrus, which contain granule neurons). (Scale bars: *A* and *B*, 200 μM; *C*, 40 μM.) (*D*) The number of hPAP$^+$ astrocytes was significantly higher in Gab/4-AP–treated than control and Gab/4-AP+TTX–treated slices, but similar to SLF-treated slices (**$p < 0.01$; $n = 17$, Kruskal–Wallis ANOVA on ranks). The data were not normally distributed and are represented as statistical box charts (horizontal lines: 25th, 50th, and 75th percentiles; error bars: 5th and 95th percentiles). (*E*) Cultures were treated as described in Fig. 1*C*. Following high K$^+$ treatment, nuclear Nrf2 protein was elevated in astrocytes that were cocultured with neuronal inserts, but not in neurons that were cocultured with astrocyte inserts; a representative of at least three similar experiments is shown. (*F*) Astrocytes were treated with either regular or neuronally preconditioned (NC) ACSF solutions for 4 h. Irrespective of the length of the conditioning step, there was no increase in nuclear Nrf2 in response to NC-high K$^+$; 16-h treatment with SLF was used as a positive control. A representative of at least three similar experiments is shown. (*G*) Experiment was performed as in *F*, except that neurons were cocultured with astrocyte inserts and astrocytes with neuron inserts for 3 d before insert removal, ACSF preconditioning, and astrocyte treatment. Again, there was no increase in nuclear Nrf2 in astrocytes treated for 4 h with NC-high K$^+$. A representative of at least three similar experiments is shown.

importantly, Gab/4-AP effect was blocked by coadministration of voltage-gated Na$^+$ channel blocker tetrodotoxin (TTX), indicating that it was mediated by increased neuronal activity (Fig. 3*D*). In agreement with these results, siRNA-mediated Nrf2 knockdown in astrocytes attenuated the increase in nuclear Nrf2 protein level induced by either Gab/4-AP or high K$^+$ treatment in direct neuron-astrocyte cocultures (Fig. S5).

To assess whether direct neuron–astrocyte contact was required for activation of astrocytic Nrf2 signaling by neuronal activity, we performed experiments in indirect neuron–astrocyte cocultures, which allow preparation of separate lysate from each cell type. Following 4-h treatment with high K$^+$, nuclear Nrf2 protein level was increased in astrocytes cocultured with neuronal inserts but not in neurons cocultured with astrocyte inserts (Fig. 3*E*), indicating that neuronal activation of the astrocytic Nrf2 pathway is mediated by diffusible messengers. Next, we examined whether Nrf2 signaling in astrocytes can be activated by high K$^+$ ACSF preconditioned on AraC-treated neurons (NC-high K$^+$); as a control, we treated astrocytes with regular high K$^+$ ACSF. Surprisingly, nuclear Nrf2 was not increased in astrocytes treated for 4 h with NC-high K$^+$ (Fig. 3*F*). In this experiment, both neurons and astrocytes were "naïve" (i.e., cultured separately), raising the possibility that coculturing neurons and astrocytes in the same medium for several days (as done in the insert experiments) leads to neuron and astrocyte changes that enable Nrf2 induction by neuronal activity. To examine this possibility, we cultured astrocytes with neuronal inserts and neurons with astrocyte inserts for 3 d before ACSF conditioning and astrocyte treatment. As in the experiment with naïve neurons and astrocytes (Fig. 3*F*), there was no increase in nuclear Nrf2 following treatment with NC-high K$^+$ (Fig. 3*G*).

Nrf2 Activation Requires Mature Synapses and Depends on Glutamatergic and Ca^{2+} Signaling. To examine whether activity-mediated up-regulation of the Nrf2 pathway requires fully mature synapses, we performed experiments in mixed cultures at different stages of maturity. Gab/4-AP treatment activated Nrf2 signaling at day in vitro (DIV)14 but had no effect at DIV3 or DIV7, suggesting that mature synapses—which form after 10 d in vitro (18)—are required for Nrf2 activation (Fig. S6*A*). To exclude the possibility that the lack of Gab/4-AP effect in early cultures was a result of low astrocyte–neuron ratio, we performed the same experiment using high K$^+$ treatment (which does not depend on fully mature synapses for its efficacy). In contrast to Gab/4-AP

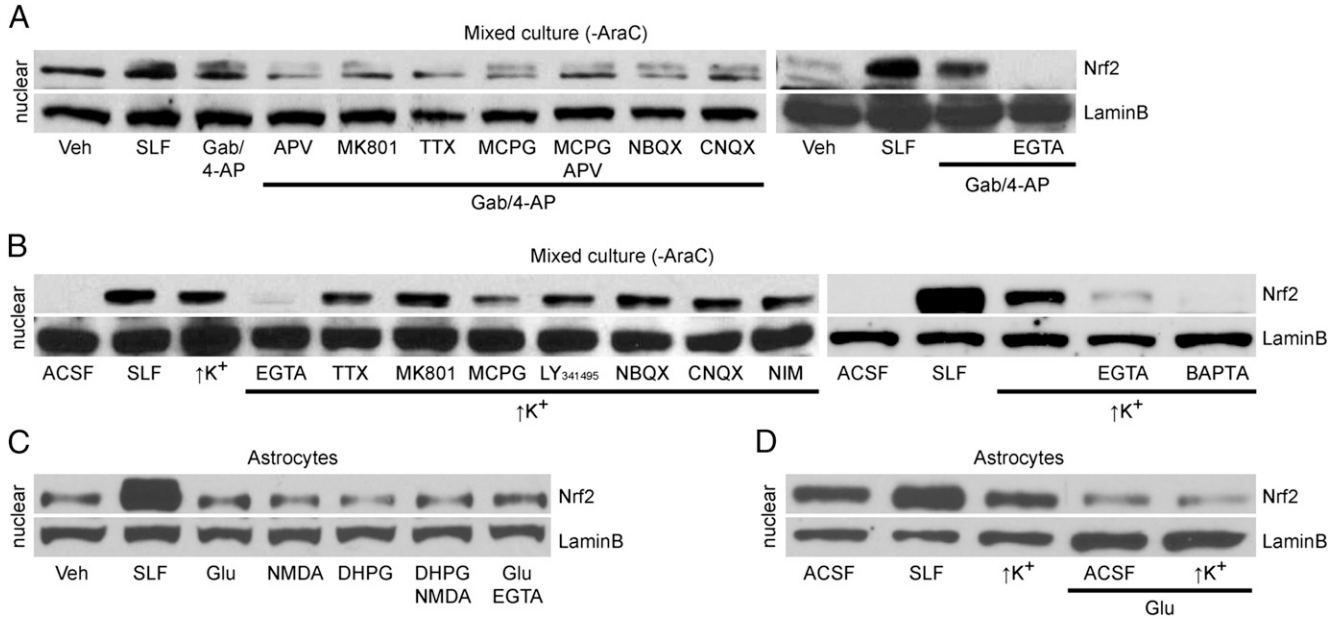

Fig. 4. Nrf2 activation depends on glutamatergic signaling and requires Ca^{2+}. (*A*) Nrf2 activation by synaptic activity was attenuated following neuronal silencing, block of metabotropic and ionotropic glutamate receptors, and chelation of extracellular Ca^{2+}. Mixed cultures were treated for 24 h with Gab/4-AP alone or with 200 μM APV, 10 μM MK801, 1 μM TTX, 50 μM MCPG, 10 μM NBQX, 10 μM CNQX, 50 μM MCPG + 200 μM APV, or 1 mM EGTA; 24-h treatment with 0.2% DMSO (Veh) was used as a negative and 16-h treatment with 2.5 μM SLF as a positive control. Representatives of four or more similar experiments are shown; for another example, see Fig. S7 *A* and *B*. (*B*) Nrf2 activation by global neuronal depolarization was attenuated following block of metabotropic (but not ionotropic) glutamate receptors and chelation of Ca^{2+}. Mixed cultures were treated for 4 h with high K^+ ACSF alone or in combination with 1 μM TTX, 10 μM nimodipine (NIM), 10 μM MK801, 200 μM APV, 1 mM EGTA, 250 μM MCPG, 1 μM LY341495 (a selective mGluR group II/III antagonist), 10 μM NBQX, or 10 μM CNQX; 4-h treatment with control ACSF was used as a negative and 16-h treatment with 2.5 μM SLF as a positive control. In BAPTA-AM experiments, cultures were pretreated with 50 μM BAPTA-AM for 30 min, washed, and then treated with high K^+ ACSF. Representatives of three or more similar experiments are shown; for another example, see Fig. S7*C*. (*C*) Astrocytes were treated for 16 h with 0.1% DMSO (Veh), 2.5 μM SLF, 300 μM glutamate (Glu), 300 μM NMDA, 10 μM DHPG (a selective mGluR group I agonist), 300 μM NMDA + 10 μM DHPG, or 300 μM Glu + 1 mM EGTA (all in culture medium); Glu or NMDA treatments were combined with 30 μM glycine, which is NMDAR coagonist. There was no increase in nuclear Nrf2 level in response to glutamate or its analogs; this outcome was seen in four of six similar experiments. Weak activation by Glu and DHPG, but not NMDA, was seen in two of six experiments (Fig. S7*D*). (*D*) Astrocytes were treated for 4 h with control or high K^+ ACSF either alone or in combination with 300 μM Glu and 30 μM glycine; 16-h treatment with 2.5 μM SLF was used as a positive control. Nuclear Nrf2 level was lower in astrocytes treated with glutamate-ACSF than in astrocytes treated with ACSF alone, but there was no difference between astrocytes treated with glutamate in control or high K^+ ACSF. A representative of four similar experiments is shown.

treatment, high K^+ treatment led to a similar increase in nuclear Nrf2 protein level at all culture ages (Fig. S6*B*), indicating that the number of astrocytes in early cultures was sufficient for Nrf2 activation.

Hippocampal cultures are primarily composed of glutamatergic neurons. To determine whether Nrf2 activation in astrocytes is mediated by neuronally secreted glutamate, mixed cultures were treated with TTX, glutamate receptor antagonists, extracellular Ca^{2+} chelator EGTA, or intracellular Ca^{2+} chelator BAPTA-AM [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] before induction of neuronal activity. When mixed cultures were treated with Gab/4-AP to selectively increase synaptic activity, increase in nuclear Nrf2 was fully or almost fully blocked by TTX, EGTA, and BAPTA (Fig. 4*A* and Fig. S7 *A* and *B*). In contrast, partial block was seen with NMDAR antagonists MK801 and APV, nonselective mGluR antagonist α-methyl-4-carboxyphenylglycine (MCPG), and AMPAR antagonists NBQX and CNQX. The results were slightly different when high K^+ treatment was used to increase global neuronal activity (Fig. 4*B* and Fig. S7*C*). As expected, under these conditions, Nrf2 activation was largely unaffected by TTX treatment; in contrast, chelation of either extracellular or intracellular Ca^{2+} completely blocked Nrf2 up-regulation. The effect of glutamate receptor antagonists was more equivocal: partial block was seen with nonselective mGluR antagonist MCPG but not with group II/III mGluR antagonist LY341495; ionotropic glutamate receptor antagonists NBQX, CNQX, APV, and MK801 were largely without effect. Finally, L-type Ca^{2+} channel blocker nimodipine had no effect on Nrf2 activation by high K^+ treatment. Although these results suggest that neuronal regulation of astrocytic Nrf2 activity is complex, it clearly depends on neuronal firing, intracellular Ca^{2+}, and glutamate receptor signaling.

To further investigate the role of glutamate receptors in the regulation of the astrocytic Nrf2 pathway, we treated astrocyte cultures with glutamate, NMDA, or 3,5-dihydroxyphenylglycine (DHPG; group 1 mGluR agonist). In the majority of our experiments, these treatments had no effect on nuclear Nrf2 level (Fig. 4*C*), although a small increase in nuclear Nrf2 was sometimes seen following glutamate and DHPG (but not NMDA) treatments (Fig. S7*D*). Finally, astrocyte cultures were treated with glutamate in high K^+ or control ACSF to investigate whether Nrf2 up-regulation requires glutamate receptor activation concurrent with astrocyte depolarization. Surprisingly, nuclear Nrf2 level was lower in astrocytes treated with glutamate-ACSF than in astrocytes treated with ACSF alone (Fig. 4*D*). Importantly, however, there was no difference in nuclear Nrf2 level between astrocytes treated with glutamate in control or high K^+ ACSF, suggesting that astrocyte depolarization has no effect on Nrf2 activity. Taken together, these data indicate that glutamate receptor activation is necessary but not sufficient for Nrf2 activation by neuronal activity.

Discussion

Neuronal susceptibility to injury critically depends on Nrf2/ARE signaling, but little is known about physiologic signals that regulate this neuroprotective pathway in the CNS. Our experiments showed that both depolarization-induced global neurotransmitter release and increased endogenous synaptic activity lead to Nrf2 activation in the mixed neuron–glia environment, but have

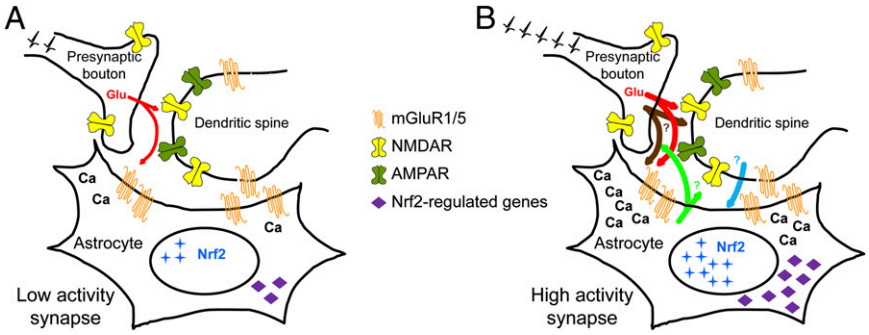

Fig. 5. Model for regulation of Nrf2 signaling at tripartite neuron–astrocyte synapse. At a low activity synapse (*A*), glutamate secreted by presynaptic terminus (red arrow) mildly activates Nrf2 signaling in adjacent astrocytes through mGluR1/5 and Ca^{2+}-dependent pathway. At a high activity synapse (*B*), increased secretion of glutamate and other (currently unidentified) soluble factors secreted by a presynaptic neuron (brown arrow), postsynaptic neuron (blue arrow), or astrocyte itself (green arrow) strongly activate astrocytic Nrf2 signaling. This regulatory mechanism matches Nrf2 activity in a perisynaptic astrocyte to the firing rate of the adjacent neuronal synapse, maximizing the neuroprotection while minimizing the associated physiologic costs.

little effect on Nrf2 signaling in predominately neuronal cultures (Fig. 1). Furthermore, we found that activity-mediated regulation of Nrf2 requires neurons and astrocytes, but not other types of glia (Fig. 2), and that activity-induced potentiation of Nrf2 signaling is restricted to astrocytes, with no up-regulation seen in neurons (Fig. 3). These findings are consistent with the previous work, which showed (*i*) that, based on gene-expression profiling, activation of synaptic NMDAR in neuronal cultures does not lead to induction of classic Nrf2-regulated genes (12); and (*ii*) that activity-dependent induction of sulfiredoxin in neuronal cultures is largely independent of Nrf2, although this gene can be up-regulated in neurons by treatment with pharmacologic Nrf2 inducers (19).

Astrocytic Nrf2 signaling was up-regulated in response to elevated neuronal activity in intact brain slices (Fig. 3 *A*–*D*), direct cocultures [which allow extensive intercellular contacts (Fig. 2 and Fig. S5)] and indirect cocultures [in which the two cell types share the same medium but are divided by a physical gap (Fig. 3*E*)]. These findings suggest that increased neuronal activity is conveyed to perisynaptic astrocytes through one or more soluble factors secreted by neurons. Surprisingly, however, there was no increase in astrocytic Nrf2 signaling in response to neuronally conditioned high K^+ ACSF (Fig. 3 *F* and *G*). There are two possible explanations for this discrepancy: (*i*) neuronally secreted factors required for Nrf2 induction are chemically unstable and thus not present at a sufficient concentration in the NC-ACSF, or (*ii*) neuronal activation of the astrocytic Nrf2 pathway requires bidirectional signaling between neurons and astrocytes.

What is the nature of soluble messengers that mediate neuronal activation of astrocytic Nrf2 signaling? When Nrf2 activation is induced by Gab/4-AP treatment, it requires a well-developed synaptic network, action potential firing, and NMDAR, AMPAR, and mGluR activity (Fig. 4*A*, and Figs. S6*A* and S7*A*). In contrast, Nrf2 activation by high K^+ treatment is seen in immature cultures with incompletely developed synapses, does not require action potential firing, and depends on metabotropic but not ionotropic glutamate receptor signaling (Fig. 4*B*, and Figs. S6*B* and S7*C*). These differences are consistent with the mechanism of action described for each treatment: although Gab/4-AP leads to network disinhibition and consequent increase in endogenous synaptic activity, high K^+ causes action potential-independent global neurotransmitter release. Irrespective of these differences, Nrf2 activation by either treatment was partly blocked by one or more glutamate receptor antagonists, indicating that neuronally secreted glutamate plays an important role in the neuronal regulation of the astrocytic Nrf2 pathway. Surprisingly, however, direct glutamate treatment was largely without an effect on astrocytic Nrf2 signaling (Fig. 4*C*), with weak activation observed only in a subset of experiments (Fig. S7*D*); this finding is consistent with the lack of Nrf2 activation by NC-ACSF, which presumably contains abundant glutamate released from presynaptic neurons during the conditioning step (Fig. 3 *F* and *G*).

Nrf2 activation by Gab/4-AP was blocked by antagonists of both ionotropic (NMDAR and AMPAR) and metabotropic glutamate receptors; in contrast, only the nonselective mGluR antagonist MCPG (but not the selective mGluR group II antagonist LY341495) partially inhibited Nrf2 activation by high K^+ (Fig. 4 and Fig. S7). Together with weak activation of astrocytic Nrf2 signaling induced by mGluR group I agonist DHPG in a subset of experiments (Fig. S7*D*), these data suggest that glutamate released by presynaptic neurons activates astrocytic Nrf2 signaling by acting on astrocytic group I mGluRs (mGluR1 or mGluR5), which couple to G_q G protein and lead to an increase in the intracellular Ca^{2+}. Ionotropic glutamate receptors, on the other hand, play a significant role only in the Nrf2 activation by synapse-dependent Gab/4-AP treatment. Together with the lack of Nrf2 activation following direct NMDA treatment of astrocytes (Fig. 4*C* and Fig. S7*D*), this finding suggests that ionotropic glutamate receptors act indirectly—at presynaptic and postsynaptic termini—by regulating neuronal release of glutamate or other secreted messengers that mediate Nrf2 activation. Collectively, these data suggest that neuronal activation of the glial Nrf2 pathway requires presynaptically released glutamate acting in combination with other factors secreted by the presynaptic neuron, postsynaptic neuron, or even the perisynaptic astrocyte in an autoregulatory feedback loop (Fig. 5); an interesting candidate for this role is superoxide anion, which is produced by neuronal NADPH oxidase in response to NMDAR activation and leads to oxidative stress in neighboring neurons and astrocytes (20).

The Nrf2 pathway can be activated through phosphorylation of Nrf2 itself or through oxidation of cysteine residues on its cytoplasmic regulator, Kelch-like ECH-associated protein 1 (Keap1) (21). Downstream of Ca^{2+}, the signal transduction pathway for Nrf2 activation thus might include Ca^{2+}-dependent kinases (such as PI3K, , Ca^{2+}/calmodulin-dependent protein kinase II, or PKC) and astrocytic NADPH oxidase, which produces reactive oxygen species in response to a rise in the cytosolic Ca^{2+} (22). Although canonical regulation of Nrf2 activity is posttranslational (2), neuronal activation of astrocytic Nrf2 signaling may also include transcriptional mechanisms: in contrast to SLF treatment, which does not alter neuronal activity (Fig. S4 *C* and *D*), both high K^+ and Gab/4-AP treatments increased Nrf2 mRNA level in mixed cultures (Fig. S8*A*); neither treatment directly altered proteasome activity (Fig. S8*C*). Interestingly, not all stimuli that elevate cytosolic Ca^{2+} in astrocytes lead to Nrf2 pathway activation: treatment of astrocyte cultures with high K^+ in the absence of neurons led to a small increase in Nrf2 mRNA level (Fig. S8*B*) but did not result in an increase in the nuclear Nrf2 protein level (Figs. 1*B*, 3 *F* and *G*, and 4*D*). Thus, either cytosolic Ca^{2+} has to be elevated within a specific signaling compartment or Nrf2 pathway activation requires simultaneous activation of two or more signaling cascades.

What is the functional significance of neuronal regulation of glial Nrf2 signaling? Elevated Nrf2 activity in astrocytes protects neurons both in vitro (7, 8) and in vivo (9, 10), and has been shown to contribute to the neuroprotective effect of ischemic preconditioning (23). Through the regulatory mechanism we uncovered, the level of Nrf2 activity in a perisynaptic astrocyte is matched to the degree of activity in the adjacent synapse (Fig. 5). Under physiologic conditions (modeled by Gab/4-AP treatment), high-activity synapses are afforded greater neuroprotection than the low activity ones; by restricting Nrf2 up-regulation only to

active synapses, this regulatory feedback loop may compartmentalize effects of Nrf2 activation on the redox-sensitive signaling pathways. Under pathologic conditions (modeled by high K^+ treatment), the synapse specificity will be lost in favor of a greater and more rapid (Fig. S1 *E* and *F*) Nrf2 activation; the globally elevated Nrf2 activity will protect the entire neuronal field at risk, albeit with possibly significant physiologic costs.

In conclusion, the activity of Keap1/Nrf2/ARE neuroprotective pathway in the CNS is fine-tuned through a regulatory loop between neurons and astrocytes: neuronal firing leads to secretion of glutamate and other (still unidentified) soluble factors, which activate the astrocytic Nrf2 pathway through a signaling cascade that involves group I mGluRs and intracellular Ca^{2+}. Uncovering the details of this regulatory pathway thus has the potential to foster development of pharmacologic treatments that will provide neuroprotection in a synapse and neuron-specific manner.

Materials and Methods

Additional experimental details (including description of Western blotting, qRT-PCR, siRNA transfection, proteasome assay, electrophysiology, hPAP histochemistry, and immunocytochemistry protocols) are provided in *SI Materials and Methods*. The use and care of animals followed the guidelines of the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Primary Mixed, Neuronal, and Astrocytic Cultures. Primary hippocampal cultures were prepared from embryonic day (E)19 embryos obtained from timed-pregnant Sprague–Dawley rats (Charles River). Following tissue dissociation, cells were cultured for 2–3 wk before experiments (unless otherwise indicated). To obtain predominantly neuronal cultures, a subset of cultures was treated with 1 μM AraC at DIV3 to prevent glial cell proliferation; mixed cultures were left untreated. Primary hippocampal or mixed hippocampal/cortical astrocytic cultures were prepared from E19 or E21 embryos; 1 wk after plating, cultures were vigorously shaken overnight to remove microglia and oligodendroglia. In all experiments, each biological replicate was performed in a different culture batch.

Direct Neuron–Astrocyte Cocultures. DIV14–28 astrocytes were plated into AraC-treated neuronal cultures (DIV10 or DIV13–14) at a density of ~1.7×10^4 cells/cm^2; experiments were done at neuronal culture DIV14–16 (i.e., either 1 or 6 d after establishment of cocultures).

Indirect Neuron–Astrocyte Cocultures. Neurons and astrocytes were cultured either in six-well tissue culture plates or on inserts designed to fit them; this experimental set-up prevents direct cell-to-cell contact but allows exchange of soluble messengers. At neuronal culture DIV14, two culture configurations were established in neuronal maintenance medium (to mimic mixed culture conditions): (*i*) astrocyte inserts were placed on top of neuronal cultures and (*ii*) neuronal inserts were placed on top of astrocytic cultures. Experiments were performed 3 d later; following treatment, only cells cultured on the bottom of six-well plates were collected for fractionation and Western blotting (cell yield from inserts was too low to obtain an adequate amount of protein). In all experiments, neuronal and mixed cultures from the same batch were used as negative and positive controls, respectively.

Quantification of $hPAP^+$ Cells. For quantification of $hPAP^+$ cells, 400-μM-thick coronal brain slices from 17 P21-26 ARE-hPAP transgenic mice were treated for 48 h with 0.1% DMSO, 20 μM Gab + 2.5 mM 4-AP, 20 μM Gab + 2.5 mM 4-AP + 1 μM TTX, or 2.5 μM SLF, and then fixed, cryoprotected, and cut into 20-μM sections for hPAP histochemistry. $hPAP^+$ cells were counted over the entire section (four to eight sections per treatment slice; one slice per animal for each treatment), with Kruskal–Wallis one-way ANOVA on ranks used for statistical analysis.

High K^+ Treatment and Preconditioning. Regular ACSF (145.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes-NaOH, 10 mM glucose, 2 mM $CaCl_2$; pH 7.4) and high K^+ ACSF (98 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes-NaOH, 10 mM glucose, 2 mM $CaCl_2$; pH 7.4) solutions were prepared fresh before each treatment. In the first set of preconditioning experiments, naïve DIV16–17 neurons (never cultured with astrocytes) were treated with control or high K^+ ACSF solution for 10 min or 4 h; preconditioned solutions were then used to treat naïve astrocytes (never cultured with neurons) for 4 h, with nonpreconditioned ACSF solutions used as a negative control. In the second set of preconditioning experiments, neurons were cocultured with astrocyte inserts and astrocytes with neuronal inserts for 3 d. After insert removal, neurons were treated with control or high K^+ ACSF solution for 10 min; preconditioned solutions were used to treat astrocytes for 4 h.

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