Distinct Subunit-specific α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptor Trafficking Mechanisms in Cultured Cortical and Hippocampal Neurons in Response to Oxygen and Glucose Deprivation**

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Background: Hippocampal CA1 neurons are more vulnerable to global ischemia than cortical neurons. Rapid internalization of GluA2 subunit contributes to OGD-induced hippocampal neuronal death.

Results: GluA2 subunit is not internalized in response to OGD in cortical neurons.

Conclusion: A crucial aspect of the mechanism leading to cell death is absent in cortical neurons.

Significance: Differences in GluA2 trafficking may contribute to neuronal vulnerability to ischemia.

Brain ischemia occurs when the blood supply to the brain is interrupted, leading to oxygen and glucose deprivation (OGD). This triggers a cascade of events causing a synaptic accumulation of glutamate. Excessive activation of glutamate receptors results in excitotoxicity and delayed cell death in vulnerable neurons. Following global cerebral ischemia, hippocampal CA1 pyramidal neurons are more vulnerable to injury than their cortical counterparts. The mechanisms that underlie this difference are unclear. Cultured hippocampal neurons respond to OGD with a rapid internalization of AMPA receptor (AMPAR) subunit GluA2, resulting in a switch from GluA2-containing Ca2+-impermeable receptors to GluA2-lacking Ca2+-permeable subtypes (CP-AMPARs). GluA2 internalization is a critical component of OGD-induced cell death in hippocampal neurons. It is unknown how AMPAR trafficking is affected in cortical neurons following OGD. Here, we show that cultured cortical neurons are resistant to an OGD insult that causes cell death in hippocampal neurons. GluA1 is inserted at the plasma membrane in both cortical and hippocampal neurons in response to OGD. In contrast, OGD causes a rapid endocytosis of GluA2 in hippocampal neurons, which is absent in cortical neurons. These data demonstrate that populations of neurons with different vulnerabilities to OGD recruit distinct cell biological mechanisms in response to insult, and that a crucial aspect of the mechanism leading to OGD-induced cell death is absent in cortical neurons. This strongly suggests that the absence of OGD-induced GluA2 trafficking contributes to the relatively low vulnerability of cortical neurons to ischemia.

AMPA receptors (AMPARs)² mediate the majority of fast synaptic excitation in the brain, and the precise regulation of AMPAR trafficking is crucial to excitatory neurotransmission, synaptic plasticity, and the consequent formation of appropriate neural circuits during learning and memory (1, 2). AMPARs are tetrameric assemblies of subunits GluA1–4, and the vast majority contain GluA2, which renders AMPARs Ca2+-impermeable (3, 4). This is a critical factor in maintaining an appropriately low cytoplasmic Ca2+ concentration during basal synaptic transmission. However, a small population of GluA2-lacking, Ca2+-permeable (CP-) AMPARs exists, which are not expressed at most synapses under resting conditions. Precise regulation of their synaptic expression allows beneficial Ca2+ signaling events, but dysregulation of these processes can lead to a prolonged synaptic incorporation of CP-AMPARs, resulting in excessive Ca2+ influx. This causes synaptic dysfunction and cell death (excitotoxicity) in a number of diseases including brain ischemia, traumatic brain injury, and chronic disorders such as Huntington disease (3–5).

Brain ischemia occurs when the blood supply to the brain is interrupted, for example by occlusion following a stroke, or as a result of cardiac arrest. The oxygen and glucose deprivation (OGD) that occurs during ischemia exposes cells to extreme metabolic stress. In neurons, this causes a breakdown of ionic gradients across the plasma membrane, resulting in depolarization and consequently a massive synaptic release of glutamate. In addition, glutamate reuptake mechanisms are compromised by transporter reversal (6). Excessive activation of glutamate receptors at excitatory synapses in the brain results in excitotoxicity in vulnerable neurons, leading to a delayed cell death (7). An important aspect of delayed neuronal death is the damage caused by reperfusion (return of oxygen and glucose to neurons after the insult). Hence, apoptotic events during and after the ischemic event may affect neuronal populations (8).

Following global cerebral ischemia, specific regions of the brain show greater neuronal injury than others, suggesting that different mechanisms that lead to cell death are recruited in response to insult. Pyramidal neurons in the hippocampal CA1
subregion are the most vulnerable, whereas CA3 pyramidal neurons are resistant (9). Although cortical pyramidal neurons are affected by ischemia, they are less vulnerable than those in hippocampal CA1 (10).

Previous studies have suggested that differential intracellular Ca\(^{2+}\) loading may play a part in this selective vulnerability, but the mechanisms that cause this difference are unclear (11). Defining these processes may lead to new therapeutic strategies based on manipulating vulnerable populations of neurons to be resistant to ischemic cell death.

The main pathway by which excitotoxicity is initiated is Ca\(^{2+}\) influx through NMDARs, which triggers a number of signaling pathways (11, 12). In hippocampal CA1 neurons, these changes in synaptic AMPAR subunit composition resulting in the expression of GluA2-lacking CP-AMPARs (13, 14). This leads to Ca\(^{2+}\) influx that contributes to delayed cell death hours to days later (15). This is a two-phase process, with an initial rapid trafficking phase involving an NMDAR-stimulated, PICK1-dependent internalization of GluA2 subunit from the plasma membrane (16, 17) and a later phase in which GluA2 subunit mRNA expression is reduced (18, 19).

Because GluA2-dependent AMPAR trafficking is an important event leading to OGD-induced neuronal death in hippocampal neurons (17), we investigated whether less vulnerable neuronal populations exhibit distinct patterns of trafficking that might contribute to the differential vulnerability to insult. We show here that GluA1 is rapidly inserted at the plasma membrane in response to OGD in both cortical and hippocampal neurons. However, in contrast to hippocampal neurons, GluA2 trafficking is unaffected by OGD in cultured cortical neurons.

**EXPERIMENTAL PROCEDURES**

**OGD**—For propidium iodide (PI) staining and surface biotinylation assays, cell cultures were exposed to OGD for 20 min as follows. Cell cultures were washed three times with HEPES-buffered saline (25 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 1.5 mM Ca\(_{\text{Cl}_2}\), 1.5 mM MgCl\(_2\), containing 15 mM sucrose for OGD condition or 15 mM glucose for control condition). OGD cultures were incubated in a hypoxic chamber (MACS-VA500 microaerophilic work station, Don Whitley Scientific) at 37 °C, 95% N\(_2\) and 5% CO\(_2\). Control cultures were incubated at 37 °C, 5% CO\(_2\) for the same time period as the OGD. After OGD the cell cultures were either returned to the original growth medium at 37 °C, 5% CO\(_2\) or immediately processed for biotinylations. For live imaging experiments, neurons were exposed to OGD by perfusion (see Live Cell Imaging).

**Propidium Iodide Staining**—PI is membrane-impermeable and excluded from viable cells. PI binds nucleic acids, enhancing its fluorescence. It therefore serves as an indicator of membrane integrity. Hippocampal and cortical neurons were exposed to 20 min of OGD and subsequently returned to their original growth medium for 0, 24, 48, and 72 h. PI (5 μM) was added to the culture medium and incubated for 10 min at 37 °C. Cells were fixed with 95% ethanol and 5% glacial acetic acid for 10 min at room temperature and subsequently stained for the neuron-specific marker, MAP-2 (Sigma-Aldrich), by immunofluorescence. Samples were imaged using a Zeiss LSM510 confocal microscope. For each of three independent experiments, three random fields of view were imaged, and all MAP2-positive neurons in these fields were analyzed. ImageJ was used to set a threshold for PI staining, which was adhered to for each experiment. The nuclei of MAP2-positive neurons with PI staining above this threshold were counted as dead cells.

**Lactate Dehydrogenase (LDH) Release Assays**—LDH release into the culture medium was determined using the LDH-based in vitro toxicity kit (Sigma-Aldrich). Neurons were exposed to 20 min of OGD and subsequently returned to their original growth medium for 0, 24, 48, and 72 h. At each time point, 0.2-ml samples of medium were taken and incubated with the LDH assay mixture according to the manufacturer’s instructions. Following reaction termination using HCl, absorbance at 492 nm was measured in a spectrophotometer.

**Surface Biotinylation**—These were performed as described (17, 20). Cortical and hippocampal cell cultures were exposed to OGD for 20 min. Cultures were placed on ice and then washed with cold PBS three times followed by incubation with 0.15 mg/ml Sulfo-NHS-SS-biotin (Thermo Scientific) in PBS for 10 min at 4 °C with gentle agitation. Cultures were washed three times with cold PBS and incubated with 50 mM NH\(_4\)Cl in PBS for 5 min at 4 °C with gentle agitation to quench excess biotin. Then cultures were washed three times with PBS and lysed in 25 mM HEPES, pH 7.4, 120 mM KCl, 1% Triton X-100, 0.1% SDS, plus protease inhibitor cocktail (Roche Diagnostics). Lysates were cleared by centrifugation, and the supernatant was incubated with streptavidin-agarose beads (Sigma) at 4 °C with rotation. After 1 h, the beads were washed three times with lysis buffer, and bound proteins were detected by Western blotting.

**Live Cell Imaging**—Cortical and hippocampal neurons were transfected at 12–13 days in vitro with plasmids expressing super-ecliptic pHluorin-tagged GluA2 (SEP-GluA2) or super-ecliptic pHluorin-tagged GluA1 (SEP-GluA1) using Lipofectamine 2000. Transfected neurons were used for experiments 4–5 days later. Imaging was performed at 37 °C using a 60× oil immersion objective of a Nikon Eclipse Ti-E microscope and Nikon confocal system C1. Neurons were continuously perfused at 37 °C with HEPES-buffered saline at a flow rate of 3 ml/min. Images were taken every 2 min at 512 × 512 resolution. To confirm that fluorescence originates from surface-expressed SEP, neurons were briefly (1 min) perfused with MES-buffered saline at pH 6 (137 mM NaCl, 5 mM KCl, 15 mM glucose, 25 mM MES, 1.5 mM Ca\(_{\text{Cl}_2}\), 1.5 mM MgCl\(_2\)). A low level of residual fluorescence could be observed in the neuronal cell body after perfusion with MES-buffered saline, corresponding to intracellular compartments with a relatively neutral pH, such as endoplasmic reticulum. A baseline was established for 10 min with normal HEPES-buffered saline perfusion prior to OGD for 20 min and reperfusion with normal HEPES-buffered saline for 20 min. All images were processed and analyzed using ImageJ software. The temporal analysis of fluorescence is calculated as ΔF/ΔF\(_0\), where ΔF corresponds to changes in fluorescence, and F\(_0\) is the average baseline fluorescence value.

**RESULTS**

It has previously been suggested that cortical neurons are less vulnerable than hippocampal CA1 neurons in a global ischemia.
model in vivo (10). To compare cell biological mechanisms activated in response to OGD, we used dissociated hippocampal or cortical cultures. Synaptic plasticity mechanisms are extensively studied in dissociated hippocampal cultures as a model for CA1 neurons because hippocampal cultures show strikingly similar cell biological properties to CA1 neurons in slices (21). PI staining demonstrates that hippocampal neurons are significantly more vulnerable to a 20-min exposure to OGD than cortical neurons (Fig. 1). Cortical cultures exhibit no detectable increase in PI staining at 24, 48, or 72 h after return to normal medium following insult. In contrast, hippocampal cultures show a significant increase in PI staining at the 48- and 72-h time points, indicating a delayed neuronal death. Moreover, similar results were obtained using LDH release assays as an alternative method for analyzing cell death (Fig. 2). These results are consistent with reports of patterns of neuronal cell death in hippocampal CA1 and cortex following global ischemia in vivo (10), and indicate that these culture systems are appropriate models for studying mechanisms that underlie differential vulnerability to ischemia.

To analyze surface expression of AMPAR subunits in cultured neurons, we used surface biotinylation assays. Using this technique, we previously showed that OGD causes a reduction in surface GluA2 in hippocampal neurons (17). To compare equivalent trafficking events in hippocampal and cortical neurons, we exposed cultured neurons to OGD and analyzed surface protein immediately after insult. In agreement with our previous results, 20 min of OGD causes a marked decrease in surface GluA2 in hippocampal neurons (Fig. 3A). In contrast, the same OGD insult has no effect on surface GluA2 levels in cortical neurons (Fig. 3A). We also analyzed surface levels of GluA1 immediately after OGD. In hippocampal neurons, there is no overall change in GluA1 surface expression, as shown previously (17). In contrast, OGD causes a significant increase in GluA1 surface expression in cortical neurons (Fig. 3B).

In some other systems such as hippocampal LTP, the presence of synaptic CP-AMPARs is transient, and CP-AMPARs are later replaced by CI-AMPARs (22, 23). To investigate the persistence of rapid OGD-induced changes in surface-expressed AMPAR subunits, cultures were exposed to OGD for 20 min and then transferred to normal conditioned growth medium for 3 h after the insult. Both cortical and hippocampal cultures exhibit the same pattern of OGD-induced changes in surface-expressed AMPAR subunits after 3 h of incubation in...
normal medium (Fig. 3, C and D). These results indicate that OGD-induced changes in AMPAR surface expression are stable and maintained well beyond the termination of the insult in both neuronal types.

In hippocampal neurons, OGD-induced AMPAR trafficking is NMDAR-dependent (17). To investigate whether a NMDAR-dependent mechanism is at play in cortical neurons, we carried out surface biotinylation experiments in the presence of the NMDAR antagonist AP5. Fig. 4 shows that AP5 completely blocks the OGD-induced increase in surface GluA1, indicating that although the pattern of subunit-specific trafficking is different in cortical neurons as compared with hippocampal neurons, both involve an upstream mechanism that is NMDAR-dependent.

To investigate the dynamic trafficking events involved in the expression of CP-AMPARs in hippocampal and cortical neurons, we used SEP-tagged AMPAR subunits in live imaging experiments (24). pHluorin is a pH-sensitive variant of GFP that is quenched at acidic pH. Hence, SEP-tagged receptors expressed on the plasma membrane are exposed to neutral pH and fluoresce brightly, whereas those localized to the acidic environment of endosomes or trafficking vesicles exhibit a dramatically reduced fluorescence (25). Hippocampal neurons expressing SEP-GluA2 show a marked decrease in fluorescence in response to OGD, indicating a rapid OGD-induced endocytosis of GluA2 from the plasma membrane, which is sustained after reperfusion with medium containing glucose and oxygen (Fig. 5A). In contrast, SEP-GluA2-expressing cortical neurons exhibit no change in fluorescence for the entire time course of the experiment, indicating that GluA2 trafficking is unaffected by OGD in cortical neurons (Fig. 5B). Interestingly, OGD-induced changes in SEP-GluA1 fluorescence are similar in hippocampal and cortical neurons, with both neuronal types showing a small but significant increase in fluorescence, indicating that OGD causes the insertion of GluA1 at the plasma membrane (Fig. 6, A and B).

DISCUSSION

Our results indicate that in hippocampal neurons, OGD stimulates a rapid GluA2-driven AMPAR endocytosis from the plasma membrane and a concomitant insertion of GluA1 into the plasma membrane from intracellular compartments. Because OGD causes no overall change in surface levels of GluA1 in hippocampal neurons, this suggests that in the native system, the endocytosed GluA2 is in the form of GluA1/2 heteromers, and that the internalized GluA1 is balanced by a GluA1-driven exocytosis of GluA2-lacking AMPAR at the plasma membrane. The exocytosed AMPARs may be GluA1 homomers or GluA1/3 heteromers (16, 26). In contrast, cortical neurons express a simpler pattern of trafficking events, whereby GluA2-dependent trafficking is completely unaffected by exposure to OGD. Although the loss of surface GluA2 persists at least 3 h after the return to normal medium in hippocampal neurons, the stability of surface GluA2 in cortical neurons is similarly maintained following reperfusion, strengthening the conclusion that GluA2-dependent AMPAR trafficking is unaffected in cortical neurons.

A number of specific forms of synaptic plasticity involve the expression of CP-AMPARs at the synaptic plasma membrane (4): for example, fear conditioning in the lateral amygdala (27), drug addiction in the ventral tegmental area (28), and hippocampal CA1 LTP, depending on developmental stage or induction protocol (22, 29, 30). In fear conditioning and drug addiction, the increase in CP-AMPAR expression is gradual, taking several hours to establish (27, 28). In contrast, hippocampal LTP involves a very rapid insertion of GluA1 at the plasma membrane, detectable immediately after the stimulus, which is similar to the trafficking we observe following initial exposure to OGD (22, 23). The increase in surface GluA1 associated with LTP is in the form of GluA2-lacking CP-AMPARs for the first 10–20 min after LTP induction, which are subsequently replaced by CI-AMPARs (22, 23, 31). Although we observe no such resetting of subunit-specific surface expression, at least up to 3 h after insult, the initial OGD-induced forward trafficking of GluA1 described here in both cortical and hippocampal neurons may share mechanistic similarities to LTP. Further work is needed to elucidate the precise signaling mechanisms and protein interaction networks involved.
Although the steady-state increase in endogenous surface GluA1 persists at least 3 h after insult in cortical neurons, our live imaging experiments suggest that homomeric SEP-GluA1 is not maintained at the surface following reperfusion with normoxic, glucose-containing medium. A possible explanation for this discrepancy is that the endogenous GluA1 is in heteromeric complexes with GluA3 and that GluA3 mediates the persistent surface expression of CP-AMPARs. Alternatively, the overexpression of SEP-GluA1 might saturate the mechanisms that retain receptors at the surface so that the increase in surface receptor number is not maintained once the driving force for forward traffic is removed. In contrast, the OGD-induced internalization of GluA2 in hippocampal neurons persists after return to normal growth medium because GluA2 is either retained in the recycling endosomal system, or more likely, ultimately targeted for lysosomal degradation. Total GluA2 expression is reduced in hippocampal CA1 after global ischemia by reduced mRNA expression (18, 19). However, lysosomal targeting could also influence total levels of GluA2 following OGD.

Our results suggest that in cortical neurons, CP-AMPARs are added to the existing complement of surface AMPARs,
whereas in hippocampal neurons, CP-AMPARs replace CI-AMPARs that are internalized via GluA2 endocytosis. We previously demonstrated that PICK1-dependent GluA2 internalization is a critical upstream component of OGD-induced cell death in hippocampal neurons (17). Our new data presented here demonstrate that GluA2 trafficking is unaffected by OGD in cortical neurons. Therefore the greater resistance of cortical neurons to OGD can be explained, at least in part, by the absence of this crucial feature of OGD-induced neuronal death. Because CP-AMPAR incorporation at the synapse would be expected to be the critical event in OGD-induced neuronal death, the precise role of CI-AMPAR internalization is unclear. A likely explanation is that GluA2-containing CI-AMPARs must be removed from the synapse to free up “slots” for CP-AMPARs. It has been shown that

**FIGURE 4.** OGD-induced increase in surface GluA1 in cortical neurons is abolished in the presence of NMDAR antagonist AP5. Cortical neurons were treated as in Fig. 3B, except AP5 was applied 5 min prior to OGD (n = 9). Data are presented as mean ± S.E.

**FIGURE 5.** SEP-GluA2 is rapidly endocytosed in response to OGD in hippocampal neurons but unaffected in cortical neurons. A, hippocampal neurons expressing SEP-GluA2 were imaged live under constant perfusion and exposed to 20 min of OGD followed by reperfusion with buffer containing normal glucose and oxygen. Representative confocal images of the time course are shown. The graph shows pooled data for fluorescence changes corresponding to SEP-GluA2 surface expression in hippocampal neurons during OGD followed by reperfusion. Post-OGD time points 20, 30, 40, and 50 min were compared with the averaged control time points (from 0 to 10 min). n = 7, *, p < 0.05, **, p < 0.01, ***, p < 0.001 (Student’s t test). Data are presented as mean ± S.E. B, cortical neurons were treated as in A. n = 7. Data are presented as mean ± S.E.
OGD causes a rapid increase in rectification of AMPAR excitatory postsynaptic currents in hippocampal neurons, indicating the synaptic expression of CP-AMPARs (17). However, the dynamic expression of CP-AMPARs at synapses on cortical neurons during or immediately after OGD has not, to our knowledge, been reported. The “slot hypothesis” suggests that PSD95-TARP complexes maintain a certain number of AMPAR positions (slots) at the synapse, and to increase the number of synaptic AMPARs, the number of slots must also increase (32). Although OGD-induced AMPAR trafficking in cortical neurons may share some common characteristics with LTP, it is unknown whether there is an increase in AMPAR slots at the synapse following insult. Following LTP induction, GluA1 is inserted into the plasma membrane at extrasynaptic sites and is subsequently incorporated into the synapse following lateral movement in the plane of the plasma membrane (33, 34). Presumably, OGD also causes an extrasynaptic insertion of GluA1. If there is no increase in the number of available synaptic slots for AMPARs, CP-AMPARs inserted at the plasma membrane may not be efficiently incorporated into the synapse. In hippocampal neurons, the internalization of CI-AMPARs results in a number of slots becoming available, allowing the incorporation of CP-AMPARs at the synapse.

In conclusion, this work suggests that GluA2-specific AMPAR trafficking mechanisms may be a suitable therapeu-

**FIGURE 6. SEP-GluA1 is inserted at the plasma membrane in response to OGD in both cortical and hippocampal neurons.** A, hippocampal neurons expressing SEP-GluA1 were imaged live under constant perfusion and exposed to 20 min of OGD followed by reperfusion with buffer containing normal glucose and oxygen. Representative confocal images of the time course are shown. The graph shows pooled data for fluorescence changes corresponding to SEP-GluA1 surface expression. Student’s t tests were applied to compare post-OGD time points (20 and 30 min) to the averaged control time points (from 0 to 10 min). n = 11, *, p < 0.05. Data are presented as mean ± S.E. B, cortical neurons were treated as in A. n = 10, *, p < 0.05. Data are presented as mean ± S.E.
tic target for manipulating neuronal susceptibility to brain ischemia.

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