Ischemia and Ischemic Tolerance Induction Differentially Regulate Protein Expression of GluR1, GluR2, and AMPA Receptor Binding Protein in the Gerbil Hippocampus

GluR2 (GluR-B) Reduction Does Not Predict Neuronal Death

Clemens Sommer, MD; Marika Kiessling, MD

Background and Purpose—Postischemic delayed neuronal death (DND) of hippocampal CA1 neurons has been suggested to occur as a result of formation of calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors lacking the GluR2 subunit (GluR2 hypothesis). DND can be prevented by a short tolerance-inducing ischemic period. The present study was designed to assess whether postischemic protein levels of GluR2 predict neuronal death. Additionally, the role of AMPA receptor binding protein (ABP) was investigated with respect to neuronal death or survival.

Methods—Postischemic protein expression of GluR1, GluR2, and ABP was analyzed in 3 experimental paradigms of transient global ischemia with the use of subunit-specific antisera and semiquantitative densitometric evaluation. Gerbils were subjected (1) to a 5-minute ischemic period resulting in DND of CA1 neurons; (2) to a 2.5-minute period of ischemia mediating tolerance induction; and (3) to 5 minutes of ischemia in the ischemia-tolerant state (2.5+5 minutes of ischemia 4 days apart).

Results—The major finding was that GluR2 protein levels were significantly downregulated in neuronal subpopulations destined to survive, ie, in CA1 principal neurons after ischemic tolerance induction and in the ischemia-tolerant state, as well as in CA3 neurons after a 5-minute period of ischemia. ABP expression remained unaffected.

Conclusions—Our results modify the GluR2 hypothesis in that postischemic GluR2 reduction also occurs in hippocampal CA1 and CA3 principal neurons without subsequent neuronal death. ABP is obviously not involved in mechanisms of DND or ischemic tolerance induction. (Stroke. 2002;33:1093-1100.)

Key Words: brain ischemia • immunohistochemistry • ischemic preconditioning • receptors, AMPA • gerbils

Received February 28, 2001; final revision received August 1, 2001; accepted August 6, 2001.
From the Department of Neuropathology, Ruprecht-Karls University of Heidelberg (Germany).
Correspondence to Dr Clemens Sommer, Laboratory of Neuropathology, Department of Pathology, University of Ulm, Albert-Einstein-Allee 11, D-89081, Ulm, Germany. E-mail clemens.sommer@medizin.uni-ulm.de
© 2002 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org

1093
tering of AMPA receptors. To address the question of whether ABP is also involved in mechanisms of DND or ischemic tolerance induction, interest in the present study also focused on a possible link between the kinetics of GluR2 protein expression and changes in ABP expression.

Materials and Methods

Animal Experiments

Experiments were performed on adult male Mongolian gerbils (Meriones unguiculatus; weight, 70 to 80 g) obtained from Charles River Deutschland (Sulzfeld, Germany). Animals had free access to food and water before experiments. Gerbils were subjected to transient forebrain ischemia by bilateral occlusion of the common carotid artery. Ischemic tolerance was induced according to the protocol of Kirino et al4 with minor modifications.25 Anesthesia was achieved with a mixture of 30% O2, 70% N2O, and 1.5% halothane. Three experimental groups of animals were investigated. One group was subjected to a single 5-minute period of ischemia, resulting in DND of CA1 neurons. The second group was subjected to a single tolerance-inducing 2.5-minute period of ischemia, and a third group was subjected to 5 minutes of ischemia in the ischemia-tolerant state (2.5+5 minutes of ischemia 4 days apart, a paradigm that results in survival of almost all CA1 neurons). Sham-operated control gerbils were subjected to anesthesia and all surgical procedures, except clamping of the carotid arteries. Animals were killed by transcardiac perfusion under deep ether anesthesia. Heparin (90 IU) was given via the left ventricle before washout of blood vessels with isotonic saline. Brains were perfusion-fixed with 4% (wt/vol) paraformaldehyde solution, removed, and postfixed overnight in the same fixative.

Neuronal cell densities within the hippocampal pyramidal layers were quantitatively assessed after labeling neurons with an antibody against the neuronal marker protein NeuN as previously described.26 Six groups of gerbils were investigated at 96 hours after sham procedure or reperfusion: untreated control gerbils (n=4), gerbils subjected to a single (n=5) or repetitive (n=3) sham operation, and gerbils subjected to 2.5 minutes (n=8), 2.5+5 minutes (n=4), or 5 minutes (n=6) of ischemia. Hippocampal subfields CA1 and CA3 of both hemispheres were scanned at a magnification of ×300. Two sets of 3 adjacent regions per area (strictly localized within the pyramidal cell layer of CA1 or CA3) per hemisphere were recorded, averaged, and expressed as mean cell number per square millimeter (neuronal density). All data were statistically analyzed with the use of the general statistics module of Analyze-it for Microsoft Excel (Analyze-it Software, Ltd). Values were expressed as mean±SEM. Significant group effects were confirmed by ANOVA and Bonferroni error protection with a significance level at P<0.05.

Immunohistochemistry

Immunohistochemistry was performed with antibodies against GluR1 protein (obtained from Chemicon International Inc), GluR2 protein (kindly provided by R. Wenthold), and ABP (kindly provided by E.B. Ziff), which were generated in rabbits immunized with bacterially expressed fusion proteins.22,28 All antibodies used are well characterized, and the specificity of antisera has been previously demonstrated.22,28–30

In Materials and Methods.

Results

Neuronal Cell Densities in the Ischemic Gerbil Hippocampus

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CA1</th>
<th>CA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>4868±227</td>
<td>2618±163</td>
</tr>
<tr>
<td>Sham (single)</td>
<td>5</td>
<td>5106±172</td>
<td>3133±208</td>
</tr>
<tr>
<td>Sham (repetitive)</td>
<td>3</td>
<td>4565±81</td>
<td>3056±116</td>
</tr>
<tr>
<td>2.5-min ischemia</td>
<td>8</td>
<td>5177±164</td>
<td>2854±64</td>
</tr>
<tr>
<td>2.5+5-min ischemia</td>
<td>4</td>
<td>4215±508</td>
<td>3299±123</td>
</tr>
<tr>
<td>5-min ischemia</td>
<td>6</td>
<td>1292±726*</td>
<td>2815±142</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, expressed as neuronal density per square millimeter.
*Values were significantly lower compared with controls, sham-operated gerbils, and gerbils subjected to 2.5- or 2.5+5-minutes ischemia (P<0.05, ANOVA and Bonferroni error protection).
these experimental groups (Table). Again, CA3 remained unaffected (Table).

**Immunohistochemistry (GluR1, GluR2, and ABP)**

**Five Minutes of Ischemia**

Prominent immunoreactivity for both GluR1 and GluR2 was present in the pyramidal layers of hippocampal subfields CA1 and CA3 in sham-operated gerbils (Figure 1). In CA1, GluR1 immunoreactivity was unchanged up to 48 hours of reperfusion and became restricted to a few scattered surviving interneurons at 96 hours (Figures 1 and 2). GluR1 immunoreactivity in CA3 remained unchanged at all time points investigated (Figures 1 and 2). Immunostaining for GluR2 in CA1 continuously decreased after ischemia, reaching significance at 48 and 96 hours. However, GluR2 immunoreactivity was not completely abolished 96 hours after reperfusion and yet was detectable in damaged shrunken CA1 neurons. In CA3, GluR2 immunoreactivity was significantly reduced between 8 and 48 hours, reattaining control levels at 96 hours after recirculation (Figures 1 and 2).

In sham-operated gerbils, ABP immunoreactivity in CA1 and CA3 was only weak in the pyramidal layer but was prominent in dendritic layers (Figure 1). Postischemic ABP immunoreactivity in CA1 was unaffected up to 48 hours after reperfusion but was significantly reduced at 96 hours (Figures 1 and 2). In CA3, no significant alterations of ABP protein expression were detectable (Figures 1 and 2).

**Tolerance-Inducing Ischemia (2.5 Minutes)**

Again, in sham-operated gerbils GluR1 immunoreactivity (not shown) and GluR2 immunoreactivity were prominent in neurons of the pyramidal layers of hippocampal subfields CA1 and CA3. A single 2.5-minute period of ischemia caused no alterations of the GluR1 immunoreactivity pattern in both CA1 and CA3 (Figure 2). GluR2 immunoreactivity in CA1, however, was transiently but significantly reduced at 24 hours (Figures 2 and 3). At 96 hours after reperfusion, GluR2 levels corresponded to those of sham-operated animals (Figures 2 and 3). Staining intensity of GluR2 immunoreactivity in CA3 was slightly but not significantly reduced between 24 and 96 hours (Figures 2 and 3).

ABP protein expression after a tolerance-inducing period of ischemia was not significantly altered in CA1 and CA3 at all time points investigated (Figures 2 and 3).

**Five Minutes of Ischemia in the Ischemia-Tolerant State (2.5+5 Minutes of Ischemia 4 Days Apart)**

In gerbils subjected to repetitive sham operation, the staining pattern and intensity for GluR1 and GluR2 corresponded to those of gerbils subjected to a single sham operation (not shown). GluR1 immunoreactivity in both CA1 and CA3 exhibited no significant alterations at all time points investigated (Figure 2). GluR2 immunoreactivity, however, showed a significant reduction at 8 hours in CA1 and was only slightly but nonsignificantly decreased at 8 and 24 hours in CA3 (Figures 2 and 4).

As after the preconditioning ischemic period, ABP immunoreactivity in the ischemia-tolerant state was unchanged in CA1 and CA3 at all time points investigated (Figures 2 and 4).

**Discussion**

Our study corroborates the GluR2 hypothesis in that, compared with GluR1, a 5-minute period of global ischemia severe enough to cause DND of CA1 pyramidal cells was associated with a continuous and selective decrease of GluR2 protein expression, which first reached significance at 48 hours after recirculation. This is a remarkable finding for several reasons. (1) In situ hybridization studies addressing postischemic GluR2 expression in CA1 yielded contradictory results: 4 studies showed a preferential decline of GluR2 mRNA in highly vulnerable CA1 neurons, whereas 3 other studies observed an unchanged ratio between the mRNAs encoding GluR1 and GluR2 subunits. (2) In contrast to global cerebral ischemia in rats with at least partially maintained postischemic protein synthesis in vulnerable CA1 neurons, total protein synthesis in the gerbil...
model of transient cerebral ischemia is almost completely and irreversibly inhibited in CA1. Despite this overall protein synthesis failure, however, heat shock protein 72 mRNA has been shown to be preferentially translated in postischemic gerbil hippocampal CA1 sector. Nevertheless, the translational deficit in CA1 neurons may have a profound impact on the respective protein expression pattern, and mRNA data alone do not predict postischemic abundance of a particular encoded protein. (3) On the protein level, Optiz and colleagues, using the same antibody as in the present study, first detected reduced posts ischemic GluR2 expression in CA1 neurons at 72 hours of reperfusion immunohistochemically and at 60 hours in microdissected CA1 sectors by immunoblotting. These data are somewhat at variance with our results. Ischemic damage of medial CA1 principal neurons precedes that of lateral pyramidal cells and frequently already manifests morphologically at 72 hours after reperfusion. At the same time point, nuclear DNA fragmentation is also

Figure 2. Semiquantitative assessment of postischemic hippocampal GluR1, GluR2, and ABP immunoreactivity in various experimental paradigms. After a single 5-minute period of ischemia, GluR2 immunoreactivity is progressively reduced in CA1 compared with GluR1 and ABP but first reaches significance immediately before DND (a). In CA3, GluR2 immunoreactivity is significantly decreased between 8 and 48 hours but has nearly reattained control values at 96 hours (b). A single 2.5-minute tolerance-inducing ischemia significantly and selectively reduces GluR2 immunoreactivity in CA1 after 24 hours (c), whereas GluR1 and ABP are unaffected in both CA1 (c) and CA3 (d). Similarly, in the ischemia-tolerant state, GluR2 is transiently reduced at 8 hours (e), whereas GluR1 is nonsignificantly decreased and ABP remains unchanged in CA1 (e) and CA3 (f). OD-sp indicates specific optical density; OD-tot, total optical density. *Significant (P<0.05, ANOVA and Bonferroni error protection).
detectable in the medial part of CA1.\textsuperscript{43} Thus, in agreement with these observations, 72 hours seems to be a critical time point beyond the threshold for incipient evidence of neuronal degeneration.

The validity of the GluR2 hypothesis has meanwhile been restricted by the original proponents to specific neuronal subpopulations. GluR2 knockdown experiments by antisense treatment led to a reduction of GluR2 in granule cells of the dentate gyrus but did not trigger granule cell degeneration, whereas neurons of CA1 and to a lesser extent neurons of CA3 underwent cell death,\textsuperscript{44} thus indicating a different sensitivity of neuronal subpopulations to GluR2 downregulation. Our present findings modify the GluR2 hypothesis in that diminished abundance of the GluR2 subunit in CA3 neurons after ischemia of 5 minutes\textsuperscript{45} duration (Figures 1 and 2) and even in the same neuronal subpopulation, ie, hippocampal CA1 neurons (at 24 hours after a single 2.5-minute period of ischemia and at 8 hours in the ischemic tolerant state), is not necessarily followed by cell death,\textsuperscript{44} thus indicating a different sensitivity of neuronal subpopulations to GluR2 downregulation. Our present findings modify the GluR2 hypothesis in that diminished abundance of the GluR2 subunit in CA3 neurons after ischemia of 5 minutes’ duration (Figures 1 and 2) and even in the same neuronal subpopulation, ie, hippocampal CA1 neurons (at 24 hours after a single 2.5-minute period of ischemia and at 8 hours in the ischemic tolerant state), is not necessarily followed by cell death (Figures 2, 3, and 4). Similarly, Friedman and colleagues,\textsuperscript{46} using the rat model of global cerebral ischemia, recently demonstrated that postischemic hypothermia does not prevent reduction of GluR2 expression in CA1 seen in normothermic animals but results in survival of vulnerable CA1 neurons 7 days after reperfusion. Kjøller and Diemer\textsuperscript{47} analyzed postischemic GluR2 protein synthesis in the rat using Western blots and a pulse-labeling approach and also detected a significant decrease of the GluR2 synthesis rate at 24 hours in vulnerable CA1 and resistant CA3 and dentate gyrus neurons. These findings corroborate our data, strongly suggesting that significantly reduced GluR2 levels are not predictive of DND in general.

Further evidence against a direct causal relationship between lack of GluR2 and neuronal death comes from experiments with transgenic animals. The GluR2(−/−) mouse is viable and has a normal complement of CA1 and CA3 neurons. Moreover, there is no evidence of neuronal degeneration in the entire central nervous system, although calcium permeability of AMPA receptors is increased up to 9-fold.\textsuperscript{48} Mice with different levels of unedited GluR2 exhibit neurological abnormalities depending on the expression levels of the allele, but no direct neurotoxic effects are detectable.\textsuperscript{47,48} In addition, hippocampal GABAergic interneurons (physiologically lacking GluR2 subunits) also do not spontaneously degenerate.\textsuperscript{49,50} According to the GluR2 hypothesis, these neurons survive because they permanently express calcium-permeable AMPA receptors, whereas only neurons that suddenly have to cope with a high calcium influx through GluR2 lacking AMPA receptors undergo cell death. However, a recent study by Tóth and McBain\textsuperscript{51} demonstrated that interneurons of the stratum lucidum constitutively possess calcium-permeable AMPA receptors but still die after transient ischemia or kainate-induced seizures.\textsuperscript{52,53} In general, the notion that postischemic calcium influx through ion- or voltage-gated calcium channels with a concomitant increase of cytosolic free calcium inevitably results in irreversible cell damage has been reexamined: Recent in vitro work indicates that depletion of endoplasmic reticulum calcium stores and/or elevated calcium uptake in mitochondria may cause neuronal death, irrespective of free cytosolic calcium concentration.\textsuperscript{54–56}

In regard to the regulation of AMPA receptor subunit expression after a preconditioning ischemic stimulus, to our knowledge there are only 3 studies, all from the laboratory of Diemer.\textsuperscript{35,45,57} Using in situ hybridization, these authors demonstrated that postischemic GluR2 expression in hippocampal CA1 neurons remains unchanged, whereas quantitative polymerase chain reaction analysis yielded a significant upregulation of GluR2 mRNA.\textsuperscript{35} The different PCR results are explained by polyadenylation of preexisting GluR2 mRNA, which in turn is thought to enhance synthesis of...
GluR2 protein, as reflected by a significant increase of GluR2 protein expression at 24 hours after preconditioning in microdissected CA1 regions assessed by immunoblotting. It cannot be excluded that the discrepancy with our immunohistochemical data, demonstrating reduced GluR2 protein expression in CA1 at 24 hours after tolerance induction (Figures 2 and 3), is due to an interspecies variation. More importantly, however, immunolabeling experiments by Western blotting do not distinguish between neuronal and nonneuronal GluR2 protein. In vitro, hippocampal astrocytes show GluR2 immunoreactivity, which may contribute to total GluR2 abundance in immunoblots. Moreover, homogenates do not reflect GluR2 subunit abundance of individual cells. To clarify this point, further experiments using in situ detection methods of GluR2 protein expression need to be performed in the preconditioned rat hippocampus.

In conclusion, in agreement with the GluR2 hypothesis, our data demonstrate that CA1 neurons destined to die after global ischemia of 5 minutes’ duration exhibit reduced levels of GluR2 protein expression immediately before cell death. However, postischemic GluR2 protein levels were also substantially and significantly reduced in neuronal subpopulations destined to survive (in CA3 neurons after 5 minutes of ischemia and in CA1 neurons after induction of ischemic tolerance and in the ischemia-tolerant state), thus further restricting and modifying the GluR2 hypothesis. ABP, a novel AMPA receptor anchoring protein, appears to be unrelated to postischemic neuronal death or survival.

Acknowledgments
This work was supported by a grant of the Deutsche Forschungsgemeinschaft (SFB 317). The authors would like to thank Robert J. Wenthold (Laboratory of Neurochemistry, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, Md) and Edward B. Ziff (Howard Hughes Medical Institute, Department of Biochemistry, New York University Medical Center, New York, NY) for kindly providing the GluR2 and ABP antiserum, respectively. The authors acknowledge the excellent technical assistance of Stephan Hennes.

References


Ischemia and Ischemic Tolerance Induction Differentially Regulate Protein Expression of GluR1, GluR2, and AMPA Receptor Binding Protein in the Gerbil Hippocampus: GluR2 (GluR-B) Reduction Does Not Predict Neuronal Death

Clemens Sommer and Marika Kiessling

*Stroke*. 2002;33:1093-1100
doi: 10.1161/01.STR.000014205.05597.45

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/33/4/1093

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/