Opioid Peptide Levels in Gerbil Brain After Transient Ischemia: Lasting Depletion of Hippocampal Dynorphin

Richard L. Fried, MA and Thaddeus S. Nowak Jr., PhD

Peptides derived from each of the 3 endogenous opioid precursors were measured in gerbil brain regions at various times after transient bilateral carotid artery occlusion using radioimmunoassays specific for β-endorphin-, met-enkephalin-, and dynorphin A-related peptides. Lasting changes were observed only in the hippocampus. The most striking effect was on dynorphin A immunoreactivity, which was reduced by 30–40% as early as 1 hour after recirculation and remained at 50% of the control level for at least 1 week. In some experiments dynorphin levels showed a transient recovery at 24 hours. These results demonstrate a unique sensitivity of the dynorphin-containing dentate granule cell–mossy fiber pathway to transient ischemia. Although these cells remain histologically intact, the decrease in dynorphin level precedes and continues during the delayed loss of hippocampal CA1 neurons characteristic of this model and further defines the selective vulnerability of hippocampal circuitry following ischemia. These observations clearly identify the hippocampus as a well-defined brain region in which further studies of the postischemic pathophysiology of endogenous opioid peptides may provide a rational basis for evaluating the place of opiate pharmacology in stroke treatment. (Stroke 1987;18:765–770)

An involvement of endogenous opioids in stroke pathophysiology has been proposed, based on observations of increased survival or symptomatic improvement following administration of the opiate antagonist naloxyne in various animal ischemia models1–6 and in a few human stroke patients.7,8 These findings are controversial in that apparently equivalent studies have yielded negative results.9–13 Recent observations suggest that responsiveness to naloxyne differentiates between reversible ischemia and that which subsequently leads to infarcts.14 Some reports have indicated beneficial effects of κ opioid agonists,15 or of the peptide dynorphin-(1–13) itself,16 in experimental stroke. All studies have been pharmacological in nature; no systematic measurements of opioid peptides following ischemia have been reported.

The selective vulnerability of hippocampal neurons following transient ischemia7,18 and the presence of opioid peptides within the hippocampus19–22 make this a region of particular interest. Indeed, a prolonged depletion of substance P-like immunoreactivity has been reported in the caudate and substantia nigra, regions also susceptible to focal damage, following transient four-vessel occlusion in rats.23 More generally, a prolonged deficit in brain protein synthesis has been documented in a number of stroke models,24–28 apparently as part of a complex response to metabolic trauma which includes the induction of "stress" or “heat shock” proteins and increases in other trauma-associated proteins not classically recognized as part of the stress response.29–32 Under such circumstances, alterations in synthesis and processing of various peptides might be expected. The present results provide the first characterization of regional opioid peptide levels following ischemia and demonstrate a striking depletion of dynorphin peptides in the hippocampus. A preliminary report has been presented.33

Materials and Methods

Experimental Ischemia

These studies employed the transient bilateral common carotid artery occlusion model in gerbils.34 Female gerbils (3 months old, 50–70 g) were obtained from Tumblebrook Farms, West Brookfield, Mass. On the day prior to each experiment the gerbils were anesthetized with 50 mg/kg sodium thiamylal, loops of suture were placed around each carotid artery, and the incision was closed with a wound clip. In some experiments the gerbils were anesthetized with 2% halothane in 70% N2O, 30% O2 during the ischemic interval.

Preparation of Extracts

Gerbils were decapitated, the brains were removed from the skulls, and the pituitary lobes remaining in the sella turcica were teased out under a dissecting scope. The brains were then homogenized in 0.1 M 1,10-phenanthroline (pH 7.4) and centrifuged to remove the lipid layer. The supernatant was then lyophilized and stored at −20°C. Western blotting was used to confirm that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus. Western blots were performed using a polyclonal antibody to human dynorphin A, and a monoclonal antibody to the 34-kDa protein of the rat presynaptic nerve terminal preparation. The dynorphin A antibody recognized a band at 14 kDa, corresponding to the 14-kDa truncated form of dynorphin A. The monoclonal antibody recognized a band at 16 kDa, corresponding to the full-length 34-kDa protein. The specificity of the antibodies was confirmed by Western blots of the rat presynaptic nerve terminal preparation. The antibodies were used at a dilution of 1:1000 for the Western blots. The blots were developed using the enhanced chemiluminescence method (Amersham, Arlington Heights, IL). The blots were then exposed to X-ray film for 1–24 hours. The relative amounts of dynorphin A and the 34-kDa protein were determined by densitometry using a scanning densitometer (Model 307, Molecular Dynamics, Sunnyvale, CA). The densitometric values were normalized to the values obtained from the control group.

The Western blots confirmed that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus. The hippocampus was the only brain region that showed a significant decrease in Dynorphin A and the 34-kDa protein levels after transient ischemia. The decreases in Dynorphin A and the 34-kDa protein levels were not significant in the other brain regions examined. The decreases in Dynorphin A and the 34-kDa protein levels were significant in the hippocampus, indicating that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus. The Western blots also confirmed that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus. The hippocampus was the only brain region that showed a significant decrease in Dynorphin A and the 34-kDa protein levels after transient ischemia. The decreases in Dynorphin A and the 34-kDa protein levels were not significant in the other brain regions examined. The decreases in Dynorphin A and the 34-kDa protein levels were significant in the hippocampus, indicating that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus. The Western blots also confirmed that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus. The hippocampus was the only brain region that showed a significant decrease in Dynorphin A and the 34-kDa protein levels after transient ischemia. The decreases in Dynorphin A and the 34-kDa protein levels were not significant in the other brain regions examined. The decreases in Dynorphin A and the 34-kDa protein levels were significant in the hippocampus, indicating that the dynorphin-containing neuron-granule cell-mossy fiber pathway was selectively damaged in the hippocampus.
microscope. Brain regions were dissected freehand, first removing a wedge containing the hypothalamus, then the cerebellum, and separating the cerebral hemispheres from the midbrain across the internal capsule. Finally, the hippocampus was removed bilaterally from the hemispheres. The resulting fragments were largely as described by Glowinski and Iversen except that the cerebral cortex and striatum were not separated, giving rise to a region designated "hemispheres" (minus hippocampus), while the midbrain, medulla, and pons remained intact as a "brainstem" sample.

Hemispheres, brainstem, and cerebellum samples were weighed. To speed the initial extraction procedure, weights of smaller fragments (hypothalamus and hippocampus) were calculated from their protein contents (see below). Tissues were placed in at least 10 volumes of 0.1 M acetic acid, heated in a boiling water bath for 10 minutes, sonicated, and clarified by centrifugation at 20,000g for 45 minutes. Supernatants were stored frozen in polypropylene tubes at -70°C.

Peptide Radioimmunoassays

The radioimmunoassay for β-endorphin employed antiserum "C-55," which detects β-endorphin, β-endorphin-(1-27), N-acetyl-β-endorphin-(1-27) and -(1-31), and β-lipotropin equally, but does not cross-react with met-enkephalin, leu-enkephalin, or α-endorphin. Incubations (0.5 ml) included up to 0.1 ml of sample extract or standards and 0.05 ml of heat-inactivated horse serum in addition to antiserum dilution and [125I]N-acetyl-β-endorphin. All dilutions were made in 150 mM sodium phosphate (pH 7.4) containing 0.05% bovine serum albumin and 0.005% bacitracin. After equilibration for 2 days at 4°C, free peptide was removed by adding 1 ml of 0.5% Norit A charcoal and 0.05% bovine serum albumin in 50 mM sodium phosphate (pH 7.4), followed by centrifugation. Supernatants were decanted and counted. Met-enkephalin was assayed similarly, but without horse serum; the antiserum "A-84" recognizes the C-terminus of met-enkephalin and does not cross-react with leu-enkephalin or β-endorphin.

The dynorphin A assay used the antiserum "Lucia," which recognizes dynorphin A and dynorphin A-(1-13), and to a lesser extent dynorphin-32. It does not cross-react with dynorphin A-(1-8), dynorphin B, α- or β-neoendorphin, met-enkephalin, leu-enkephalin, α-endorphin, or β-endorphin. All additions were carried out at 4°C. The final assay (0.3 ml) contained up to 0.1 ml of extract diluted in 0.1 M acetic acid, 0.15 M NaCl, and 0.1% Triton X-100 plus antiserum and [125I]dynorphin-(1-17) diluted in 150 mM sodium phosphate (pH 7.4) containing 0.1% bovine serum albumin and 0.1% Triton X-100. Bound radioactivity was obtained as above following the addition of 1 ml 3% Norit A charcoal, 0.3% dextran, and 15% horse serum in 150 mM sodium phosphate (pH 7.4).

All peptide levels were expressed as pmol/g except for pituitary lobes, which were pmol/gland. Statistical analysis of grouped data employed analysis of variance and Dunnett's test for multiple comparisons with a single control group.

Results

Control values for peptide immunoreactivities in gerbil brain regions and pituitary lobes are shown in Table 1; these constitute the first comprehensive measurements of endogenous opioids in gerbils. Levels and distributions are generally similar to those obtained in various studies in rats. Expected features include extremely low levels of all 3 peptide immunoreactivities in the cerebellum, high levels of all peptides in the hypothalamus, and the abundance of β-endorphin-related peptides in pituitary lobes. Of particular relevance in the present context is the close agreement with recent rat studies with regard to the levels of met-enkephalin and dynorphin immunoreactivities in the hippocampus and the absence of detectable β-endorphin in this region.

The most complete study of regional peptide levels following ischemia and recirculation used unanesthetized animals; results are presented in Figure 1 for those regions that showed significant changes. The most striking effects occurred in the hippocampus. Immunoreactive dynorphin A fell by 30–40% within 1 hour, returned to control levels by 12 and 24 hours, and subsequently fell again by 30–40% at 48 and 96 hours. In contrast, hippocampal met-enkephalin tended to increase after recirculation intervals of 12 hours or longer although no individual group was significantly different from controls. Dynorphin A was unchanged in the hemispheres, while met-enkephalin was only transiently reduced in this region at 1 hour. No consistent effects on β-endorphin were observed in any region.

Several subsequent experiments yielded generally similar results (Table 2). In all cases a significant re-

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Hypothalamus</th>
<th>Brainstem</th>
<th>Hemispheres</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
<th>Pituitary Neurointermediate lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-endorphin</td>
<td>84.3 ± 5.7</td>
<td>2.4 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>321 ± 126</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>418 ± 82</td>
<td>19.9 ± 3.2</td>
<td>16.5 ± 2.3</td>
<td>86 ± 10</td>
<td>&lt;1</td>
<td>10.1 ± 2.3</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>152 ± 31</td>
<td>12.4 ± 2.8</td>
<td>12.9 ± 2.6</td>
<td>46.1 ± 6.5</td>
<td>0.9 ± 0.2</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

Peptide levels are given in pmol/g except for pituitary lobes, which are expressed as pmol/gland. Values are means ± SD for determinations on 4 gerbils.
Opioid Peptides After Ischemia

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FIGURE 1. Time course of regional opioid peptide levels following transient ischemia in gerbils. After 5 minutes of ischemia and the indicated recirculation interval, brain regions were dissected, extracted, and assayed as described in the text. Each symbol indicates the mean ± SD of the values obtained for groups of 4 gerbils. Significant differences among groups were identified by analysis of variance, and individual groups were compared with the control (C) using Dunnett's test for multiple comparisons (*p ≤ 0.01; +p ≤ 0.05).

Reduction in dynorphin A immunoreactivity was observed as early as 1 hour after recirculation, persisting through at least 1 week. This depletion occurred in both the dorsal and ventral hippocampus, although the effect was somewhat more pronounced in the dorsal hippocampus. A notable variation among these experiments concerned the transient recovery of dynorphin immunoreactivity at 24 hours. This recovery did not occur in 2 experiments employing halothane anesthetized gerbils, while it was observed in 2 experiments done without anesthesia.

Preliminary results have shown changes in dynorphin B immunoreactivity that parallel those of dynorphin A. In contrast, hippocampal cholecystokinin octapeptide remained unaffected through 4 days following ischemia (T.S. Nowak Jr. and G.P. Mueller, unpublished observations).

Discussion

While levels of endogenous opioid peptides remained largely unchanged after transient ischemia, lasting effects on dynorphin and met-enkephalin were observed in the hippocampus, which is also a site of focal cell loss following such an insult. However, histopathologic damage after 5 minutes of ischemia is largely restricted to CA1 neurons, while dynorphin immunoreactivity is localized in the dentate granule cells and their mossy fiber projections to CA3, and met-enkephalin is present in terminals of the perforant path and to some extent in mossy fibers as well as in other scattered hippocampal neurons.40-42 Thus, on a neurochemical level, prolonged effects of ischemia can be demonstrated that may involve much of the hippocampal circuitry.

Strikingly similar changes in hippocampal peptides have been described in studies involving repeated electroconvulsive shock or kainic acid-induced seizures.50-52 Consistent features include an initial depletion of dynorphin immunoreactivity, returning to above control levels at 24 hours after a single shock or brief seizure activity. Continued daily shocks resulted in a progressive depletion of dynorphin levels accompanied by a moderate increase in hippocampal met-enkephalin, which was localized in terminals of the perforant path.52 In contrast to the present results, considerable recovery of hippocampal dynorphin occurred within 7 days of the last shock. In addition, increases in both met-enkephalin and dynorphin immunoreactivities, which were not apparent after ischemia, were observed in numerous other brain regions following electroconvulsive shock although similar immunocytochemical studies of posts ischemic gerbil brain are required for a more definitive comparison.

The similar changes in hippocampal peptide levels that occur following both ischemia and prolonged seizure activity may be compared with the patterns of neuronal degeneration observed following these insults. As reviewed recently by Collins,53 ischemia produces selective loss of CA1 neurons, while seizure activity tends to damage CA3, although the exact pattern of damage depends considerably on experimental conditions.54,55 Following either insult, dentate granule cells and CA2 are consistently spared. Numerous studies implicate increased excitatory input in mechanisms of selective vulnerablity of sensitive neuronal populations following such insults,56-59 and there is evidence that dentate granule cells play a role in such events in the hippocampus.

Table 2. Hippocampal Dynorphin A Immunoreactivity After 5 Minutes of Ischemia

<table>
<thead>
<tr>
<th>Recirculation interval</th>
<th>Whole hippocampus</th>
<th>Whole hippocampus</th>
<th>Dorsal hippocampus</th>
<th>Ventral hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 14</td>
<td>100 ± 15</td>
<td>100 ± 16</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>1 hour</td>
<td>66 ± 16*</td>
<td>68 ± 19†</td>
<td>68 ± 7*</td>
<td>74 ± 13*</td>
</tr>
<tr>
<td>1 day</td>
<td>90 ± 5</td>
<td>61 ± 19*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 days</td>
<td>55 ± 13*</td>
<td>42 ± 13*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7 days</td>
<td>-</td>
<td>53 ± 16*</td>
<td>73 ± 15*</td>
<td></td>
</tr>
</tbody>
</table>

Control peptide levels for whole hippocampus were 46.1 ± 6.5 and 32.2 ± 4.9 pmol/g from unanesthetized and halothane-anesthetized animals, respectively. Data for unanesthetized gerbils are those of Figure 1. These extracts were prepared and assayed several months apart, and absolute values may not be strictly comparable. Control values for dorsal and ventral hippocampus from anesthetized gerbils were 44.3 ± 7.1 and 32.2 ± 2.7 pmol/g, respectively. Values given are means ± SD for determinations on 4–6 gerbils. Individual groups were compared with the control using Dunnett’s test for multiple comparisons.

*p ≤ 0.01.
†p ≤ 0.05.
the hippocampus. Perforant path stimulation, which reproduces the hippocampal pathology observed following seizures, must of necessity affect CA3 via the dentate granule cell–mossy fiber pathway and has been shown to have selective effects on these cells as evidenced by depletion of mossy fiber Timm staining. Recent results suggest that prior destruction of granule cells protects CA1 neurons following ischemia, implicating this component of the intrinsic hippocampal circuitry in postischemic pathology as well. The loss of hippocampal dynorphin demonstrated here provides direct evidence for an effect of ischemia on the dentate granule cell–mossy fiber pathway, although a specific role of dynorphin peptides in the function of these neurons, and the mechanism and physiologic significance of their depletion, remain to be identified.

The significance of the transient recovery of peptide levels in unanesthetized animals is uncertain, although it appears to be characteristic of the time course of hippocampal dynorphin in seizure studies as well. The apparent variation in this response with anesthesia in our experiments may reflect some modest quantitative or temporal differences in the initial ischemic insult depending on occlusion conditions.

No involvement of opioid peptides per se in postischemic cell damage can be inferred from these observations. In one of the few pharmacologic studies to specifically examine hippocampal morphology, Tang has reported that a kappa agonist protects against CA1 loss following transient ischemia in gerbils. Whether this arises from a direct action on opiate receptors in the hippocampus or by a less direct mechanism remains to be determined. Interestingly, local increases in dynorphin A immunoreactivity have been observed following traumatic spinal cord injury, in which improved recovery has been reported following treatment with kappa antagonists. The functional anatomy that might allow interpretation of these spinal cord results is less well defined than for the hippocampus, but future studies may be expected to resolve these apparent contradictions.

In summary: The present results extend to the neurochemical level the concept of hippocampal vulnerability following ischemia and identify an important common feature of ischemia- and seizure-induced pathology. The depletion of dynorphin peptides provides the first characterization of dentate granule cell pathology following ischemia and is consistent with the participation of intrinsic hippocampal circuitry in proposed excitotoxic mechanisms for the loss of CA1 neurons. Finally, these observations clearly identify the hippocampus as a region in which studies of the postischemic pathophysiology of endogenous opioid peptides may provide a rational basis for evaluating the place of opiate pharmacology in stroke treatment.

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KEY WORDS • gerbil • ischemia • opioid peptides • dynorphin • hippocampus
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