Electrical stimulation in vivo increases the expression of proenkephalin mRNA and decreases the expression of prodynorphin mRNA in rat hippocampal granule cells

Brian J. Morris*,†, Karin J. Feasey‡, Gerrit ten Bruggencate‡, Albert Herz*, and Volker Hölzl†‡§

*Department of Neuropharmacology, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18a, D-8033 Planegg-Martinsried, Federal Republic of Germany; and ‡Physiologisches Institut, Universität München, Munich, Federal Republic of Germany

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ABSTRACT In situ hybridization histochemistry in combination with RNA blot techniques was used to study the regulation of opioid gene expression in rat hippocampus. By use of a prodynorphin cDNA probe, a strong hybridization signal was identified in the granule cell layer of the hippocampus. However, experiments using a proenkephalin cDNA probe revealed that the content of proenkephalin mRNA was considerably lower than that of prodynorphin mRNA. Following five brief trains of high-frequency electrical stimulation to the dentate gyrus, the proenkephalin mRNA content of the granule cells, measured 22 hr later, was substantially increased on the stimulated side. In contrast, levels of prodynorphin mRNA were markedly decreased ipsilaterally to the stimulation site. These results were confirmed by RNA blot analysis of extracted mRNA. The decrease in prodynorphin mRNA content first appeared between 4 and 7 hr after the end of stimulation. Distinct mechanisms, therefore, regulate the expression of proenkephalin mRNA and prodynorphin mRNA in rat hippocampus.

A fundamental question in neurobiology is how the level of gene expression within neurons is regulated by the electrical and neurochemical signals they receive. Changes in the expression of neurotransmitter and related gene products alter alterations in neuronal activity would represent a mechanism for neural plasticity.

In the simplest model, depolarization of the cell membrane leads not only to neurotransmitter release, but also to an enhanced rate of neurotransmitter synthesis. Depolarization of cells in culture has been shown to increase activity of the enzymes that synthesize the classical neurotransmitters (1–3) and to increase the levels of mRNA encoding peptides neurotransmitters (4–7). However, that depolarization can depress levels of peptide precursor mRNAs in cell culture has also been indicated (8, 9), suggesting the existence of complex control mechanisms.

To understand how neurotransmitter gene expression is regulated in particular brain regions, cells must be studied in their normal neuroanatomical environment with afferent and efferent inputs intact, rather than in cell culture. Recent studies in our laboratories have focused on the regulation of neurotransmitter gene expression in the hippocampus (10), a region where long-term changes in synaptic efficacy are readily demonstrated and probably play a role in the molecular basis of memory (11, 12). The granule cells of the hippocampal dentate gyrus contain peptides derived from both proenkephalin and prodynorphin (13, 14), in addition to high levels of prodynorphin mRNA (15, 16). We now report the effect of direct electrical stimulation of the granule cells in vivo on the levels of proenkephalin mRNA and prodynorphin mRNA.

MATERIALS AND METHODS

In Vivo Stimulation. Experiments were done on male Sprague-Dawley rats (250–300 g) anesthetized with Nembutal (60 mg/kg, i.p.). A bipolar concentric stimulating electrode was stereotaxically placed in the dentate gyrus of the left hippocampus (2.5 mm lateral, 3.8 mm posterior to the bregma, 3.5 mm deep to the cortical surface).

Five high-frequency stimulation trains (250 Hz, 250 ms, 50 V) were delivered at 20-min intervals. After the last train, the stimulating electrode was removed, and the incision was closed with suture.

Sham-operated animals were treated identically, except that after placement of the stimulating electrode, no stimuli were delivered. Animals were killed 4 hr, 7 hr, or 22 hr after delivery of the last high-frequency train.

In Situ Hybridization. Cryostat sections (10 μm) were mounted onto polylysine-coated slides, dried, and fixed for 15 min in 4% (vol/vol) paraformaldehyde as described (10, 16). After treatment with 0.2 M HCl (10 min), proteinase K at 1 μg/ml (15 min), 0.25% acetic anhydride (2 min), and 4% paraformaldehyde (5 min), sections were prehybridized for 1.5 hr in 5× Denhardt’s solution (1× Denhardt’s solution is 0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.02% Ficoll)/salmon sperm DNA at 0.5 mg/ml/5× SSC (1× SSC is 0.15 M NaCl/0.015 M trisodium citrate, pH 7.0)/50 mM sodium phosphate buffer, pH 7.0/50% (vol/vol) deionized formamide. The slides were then dehydrated in ethanol and hybridized for 24 hr with the radiolabeled probe in prehybridization buffer with added yeast tRNA (10 mg/ml). With the prodynorphin cDNA probe, the buffer also contained 6% (wt/vol) dextran sulfate.

For determination of proenkephalin mRNA, in situ hybridization was done using a 32P-labeled nick-translated Smal I–Sac I restriction fragment representing ~1000 bases of rat proenkephalin mRNA sequence (17, 18). Probe specific activity was 2 × 106 cpm/μg, fragment size was in the region of 60–80 bases, and hybridization was at 45°C. For determination of prodynorphin mRNA, a 100-base cDNA oligonucleotide was synthesized and radiolabeled using 32P-ATP and Klenow fragment along with a synthetic oligonucleotide template and primer, as previously described (16). The probe corresponds to nucleotides 488–587 of rat prodynorphin mRNA (15). Probe specific activity was 107 cpm/μg, and hybridization was at 50°C. Sections were washed under conditions of increasing stringency (2× SSC, 1× SSC, and

1Present address: Medical Research Council Molecular Neurobiology Unit, Hills Road, Cambridge, U.K.
2To whom reprint requests should be addressed.

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0.1 x SSC for proenkephalin cDNA probe; 6 x SSC, 3 x SSC, and 1 x SSC for prodynorphin cDNA probe, total time 3 hr) at the hybridization temperature, dehydrated in ethanol, and apposed to Cronex MRF 32 film (DuPont). Densitometry was done using a Leitz Texture Analysis System with microscope-linked densitometer. OD values are expressed as the remainder following subtraction of tissue background OD.

RNA Blot Analysis. Hippocampal tissue was dissected out and frozen on dry ice, and total RNA was then extracted using lithium chloride (19). Aliquots of 5 μg of RNA were denatured with glyoxal, run on a 1.2% agarose gel, and transferred to nylon sheets (Nytran, Schleicher & Schuell). Filters were baked for 2 hr at 80°C, prehybridized, and hybridized with either proenkephalin RNA or prodynorphin RNA probes at 50°C. These probes were obtained by subcloning the cDNA sequences into the Bluescribe vector (Stratagene, San Diego, CA), which allows single-strand sense or antisense RNA transcript to be prepared using T3 or T7 RNA polymerase. The 1000-base proenkephalin cDNA sequence and the 75-base prodynorphin cDNA oligonucleotide after second-strand synthesis were both cloned into this vector. Filters were then washed, dried, and exposed to x-ray film. Autoradiograms were scanned using a laser densitometer (LKB).

Peptide RIA. Right and left hippocampi were dissected out and processed for RIA as described (20) with antisera raised against [Met]enkephalin or dynorphin A. Antiserum cross-reactivity has been documented elsewhere (20, 21).

RESULTS

Effect of Electrical Stimulation. In unoperated and sham-operated animals, a strong hybridization signal was seen with the prodynorphin cDNA probe over the granule cell layer of the hippocampus (Fig. 1A), whereas no signal could be discerned in other regions of the hippocampus. In contrast, no signal was seen in the hippocampus after hybridization with the proenkephalin cDNA probe (Fig. 1C), consistent with the comparatively low levels of proenkephalin mRNA in this tissue (15, 18, 22-24).

In animals that had received unilateral electrical stimulation of the dentate gyrus, ipsilateral granule cell content of prodynorphin mRNA decreased markedly after 22 hr (Fig. 1B); signal in the contralateral hippocampus appeared unaffected. However, content of proenkephalin mRNA appeared to be substantially increased in the stimulated granule cells, and in some animals a slight increase was also apparent on the contralateral side (Fig. 1D).

When RNA was extracted from stimulated and nonstimulated hippocampi and the RNA blots sequentially hybridized with the proenkephalin followed by the prodynorphin cRNA probes, a single band was seen with a size corresponding to 1450 bases with the proenkephalin probe and to 2600 bases with the prodynorphin probe—the sizes expected for the mRNA species (15, 17, 18). A marked increase in hybridizable proenkephalin was found on the stimulated side compared with the nonstimulated side (Fig. 2 Lower, Table 1). No alterations were seen in levels of either mRNA in sham-operated animals.

To investigate the time course of the decrease in hippocampal prodynorphin mRNA, rats were killed 4, 7, and 22 hr after unilateral stimulation. No alteration in prodynorphin mRNA was seen after 4 hr, and the effect first became evident 7 hr after stimulation (Fig. 3).

Quantitative densitometry of the in situ hybridization and the RNA gel data obtained 22 hr after electrical stimulation

![Fig. 1](image_url)

Fig. 1. Effect of unilateral electrical stimulation on levels of prodynorphin mRNA and proenkephalin mRNA in rat hippocampus. Sections were taken from animals 22 hr after a sham operation (A and C) or 22 hr after stimulation of the dentate gyrus on the right side of the autoradiograph (B and D). Adjacent sections from each animal were hybridized with a prodynorphin cDNA (A and B) or proenkephalin cDNA (C and D) probe. Note decrease in prodynorphin mRNA and corresponding increase in proenkephalin mRNA on the stimulated side. (Exposure time, 28 days; ×4.8.)
is shown in Table 1. Autoradiographic grain densities over the dentate gyrus were unchanged in nonstimulated, sham-operated rats after in situ hybridization with the prodynorphin probe, whereas a 60% decrease in grain density was seen on the stimulated side relative to the nonstimulated side. A similar decrease in levels of prodynorphin mRNA in the stimulated, relative to the nonstimulated, hippocampi was obtained by densitometric scanning of the 2600-kb bands of the RNA blots. In contrast, in the same RNA samples, proenkephalin mRNA of stimulated hippocampi increased >3-fold compared with contralateral counterparts. The same RNA gel data were obtained in an additional experiment in which the RNA was extracted using the guanidinium/cesium chloride method (data not shown). The marked increase in the proenkephalin mRNA in granule cells of the stimulated den-
tate gyrus allowed densitometric quantification of the in situ experiments, whereas the amount of mRNA in nonstimulated hippocampi was too low to be measured with sufficient precision by this technique (Fig. 1C, Table 1). As revealed by analysis of RNA blots, there appear to be no major changes in opioid peptide mRNA levels in the nonstimulated side of the hippocampus as compared to that in hippocampi from sham-stimulated animals (Table 1).

We then asked whether electrical stimulation affected the tissue levels of peptides derived from prodynorphin and proenkephalin. In contrast to opioid peptide mRNA levels, level of immunoreactive dynorphin A or immunoreactive [Met]enkephalin did not change significantly between stimulated and nonstimulated sides of the hippocampi, although levels of both peptides on the side of stimulation did increase slightly. In addition, a small but significant increase in levels of immunoreactive [Met]enkephalin (but not of immunoreactive dynorphin A) occurred in both sides of the hippocampi of stimulated as compared with that of sham-stimulated rats (Table 1).

### DISCUSSION

The various control experiments detailed here all provide evidence that, under the conditions used for in situ hybridization, only authentic prodynorphin mRNA or proenkephalin mRNA is being detected. Because the sensitivity of the in situ hybridization technique is markedly higher when cDNA probes, rather than cRNA probes, are used (B.J.M. and V.H., unpublished data), the use of cRNA probes was here

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Table 1. Effect of unilateral electrical stimulation on the hippocampal levels of prodynorphin mRNA, proenkephalin mRNA, dynorphin A immunoreactivity, and [Met]enkephalin immunoreactivity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham-operated</th>
<th>Stimulated</th>
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<tbody>
<tr>
<td><strong>In situ mRNA, OD unit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prodynorphin mRNA</td>
<td>137.0 ± 30.9</td>
<td>124.8 ± 15.9</td>
</tr>
<tr>
<td>Proenkephalin mRNA</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
<tr>
<td><strong>RNA blot, OD × 10^6</strong></td>
<td></td>
<td></td>
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<tr>
<td>Prodynorphin mRNA</td>
<td>2.92 (2.84; 3.00)</td>
<td>3.25 ± 0.25</td>
</tr>
<tr>
<td>Proenkephalin mRNA</td>
<td>1.14 (1.01; 1.27)</td>
<td>0.83 ± 0.19</td>
</tr>
<tr>
<td><strong>Peptide, pmol/g of tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynorphin A-ir</td>
<td>59.4 ± 3.6</td>
<td>59.8 ± 2.5</td>
</tr>
<tr>
<td>[Met]enkephalin-ir</td>
<td>95.0 ± 2.8</td>
<td>129.8 ± 7.5^t</td>
</tr>
</tbody>
</table>

- ir, Immunoreactive. One-digit numbers in parentheses represent n; pair of numbers in parentheses represents numbers averaged from two experiments. Most values indicate ± SEM. Due to low basal levels, proenkephalin mRNA could be reliably measured only in electrically stimulated hippocampi.

<P> < 0.05 compared with contralateral side.

\[ P < 0.05 \] compared with corresponding sides of hippocampi of sham-operated rats (Student’s two-tailed t test).
restricted to control in situ hybridization experiments and RNA analysis. Evidence from both in situ hybridization and in vitro RNA blot analysis revealed a clear increase in proenkephalin mRNA ipsilaterally after stimulation of the dentate gyrus. Apart from general evidence linking cell depolarization with enhanced biosynthetic activity, this result is consistent with reports that epileptiform activity in the hippocampus, induced either by amygdaloid kindling (27), hilar lesion (28), or intrastriatal kainic acid (29) elevates hippocampal proenkephalin mRNA levels.

The ipsilateral suppression of prodynorphin mRNA levels is, however, unexpected. That the decrease is due to neuronal damage appears unlikely because Nissl-stained sections showed no evidence of cell loss and because the content of proenkephalin mRNA in the granule cell layer increases after stimulation. As noted above, there are examples from in vitro systems where depolarization leads to decreased hormonal or neurotransmitter mRNA levels. Thus, in the rat dentate gyrus expression of the prodynorphin gene at the mRNA level may be negatively regulated by cell stimulation.

This hypothesis clearly implies an opposite control of prodynorphin and proenkephalin gene expression in the dentate gyrus. A divergence has been noted at both peptide and mRNA level in the response of hippocampal enkephalinergic and dynorphinergic neurons to kindled seizures (10, 27, 30, 31). Because some evidence exists that enkephalin- and dynorphin-related peptides might have opposite effects on hippocampal pyramidal neurons (32, 36), these contrasting responses might fulfill a complementary function in modulating hippocampal excitability.

The mechanism underlying stimulation-induced suppression of dentate prodynorphin mRNA levels remains to be determined. Although the stimulation may activate local inhibitory interneurons and prodynorphin gene expression in the granule cells may be particularly sensitive to these inhibitory effects, it is also possible that the second messengers activated in the granule cells directly suppress prodynorphin gene expression while enhancing proenkephalin gene expression.

Time course of the decrease in prodynorphin mRNA levels, occurring over 4–22 hr poststimulation, is similar to that reported for other alterations in nervous tissue mRNA content (29, 37). The fact that prodynorphin mRNA levels fall implies that prodynorphin synthesis is decreased. Consequently, the absence of any concurrent decrease in the levels of immunoreactive dynorphin A ipsilateral to the stimulation after 22 hr suggests (i) that dynorphin release is also reduced over this period and, therefore, (ii) that the activity of the dynorphinergic component of the mossy fiber pathway is suppressed after granule cell stimulation.

A concomitant increase in release of proenkephalin-derived peptides might also explain the unaltered (or only slightly increased) tissue levels of immunoreactive [Met]enkephalin, despite the marked increase in biosynthesis of proenkephalin. However, upon repeated hippocampal stimulation as used in kindling, pronounced alterations in levels of both dynorphin- and [Met]enkephalin-immunoreactivity have been seen (27, 30, 31, 38). Therefore, the degree of alteration of peptide levels probably reflects the severity and duration of the stimulation procedure. Moreover, for enkephalin-related peptides the situation is complicated by the presence of a substantial extrinsic enkephalinergic innervation via the perforant path (39). Such factors, in addition to considerable evidence supporting mRNA levels as a reliable index of peptide turnover, emphasize the advantages of studying mRNA levels rather than peptide levels for estimating changes in peptidergic neuronal activity.

These results therefore suggest that subsequent to stimulation of the dentate gyrus, activity of the enkephalinergic projection increases, which probably has excitatory effects (32, 33, 40), and activity of the dynorphinergic projection decreases, which may have mainly inhibitory actions (34–36).

Should this be the case, this combination of effects might be expected to enhance the response of these postsynaptic neurons to ensuing afferent excitation. The stimulation parameters used here can induce long-term potentiation in hippocampal pyramidal cells and granule cells (41, 42). In contrast to the relative agreement on mechanisms responsible for postsynaptic induction of long-term potentiation, there is little consensus on factors involved in maintenance of long-term potentiation (12), although presynaptic mechanisms have been proposed (42–44). The results reported here raise the intriguing possibility that the sustained alterations in opioid gene expression in granule cells might contribute to maintenance of long-term potentiation in the pyramidal cells.

In addition, these stimulation-induced changes in neuropeptide gene expression may represent a more general phenomenon contributing to long-term changes in synaptic efficiency in other central nervous system pathways.
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