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Selective alterations of RNA in rat hippocampus after entorhinal cortex lesioning

(mRNA prevalence/deafferentation/synaptogenesis/two-dimensional gel)

J. POIRIER*†, P. C. MAY*, H. H. OSTERBURG*, J. GEDDES‡, C. COTMAN‡, AND C. E. FINCH*

*Andrus Gerontology Center and Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089; and ‡Department of Psychobiology, University of California at Irvine, Irvine, CA 92717

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ABSTRACT **_In vitro_ translation products from RNA of rat hippocampus after deafferentation by entorhinal cortex lesions were analyzed by two-dimensional gel electrophoresis. Although hippocampal total RNA yield was not affected 14 days after the lesion, analysis of the gels showed reproducible changes in the steady-state level of several transcripts. Glial fibrillary acidic protein RNA increased 2-fold over control hippocampi RNA. Moreover, seven other transcripts of unknown identity had increased prevalence in the denervated hippocampus. The changes, which ranged from 2- to 20-fold, involved mRNA encoding small slightly acidic polypeptides: 12 kDa (pI 5.6), 13 kDa (pI 6.1), 20 kDa (pI 5.8), 31 kDa (pI 5.7), 33 kDa (pI 5.7), 35 kDa (pI 5.6), and 53 kDa (pI 5.4). These results suggest new molecular markers for analyzing the complex mechanisms of synaptic reorganization in the dentate gyrus after deafferentation.**

Partial deafferentation in selected areas of the adult rat brain induces a sequence of compensatory events in both the remaining afferent fibers and denervated dendrites, resulting in a reorganization of the circuitry in the denervated area. The regeneration events include "sprouting" of the remaining afferent fibers, restoration of spine density and length, and replacement of vacated synaptic contacts. This phenomenon has been studied most thoroughly in the hippocampus, particularly for changes in the dentate gyrus in response to the loss of inputs after entorhinal cortex lesion.

The dentate gyrus of the rat hippocampal formation manifests extensive rearrangement and regrowth of residual neuronal circuitry after entorhinal cortex lesions (ECLs) (1–3). Under defined conditions, ECL disrupts the perforant path, thereby removing cortical connections to the hippocampus and causing loss of nearly 60% of the synaptic input to the granule cell layer. The loss of synapses, however, is transient. Beginning a few days after denervation, new synapses are formed, virtually replacing the lost inputs within 2 months. These new synapses originate from the cholinergic septal neurons (3), glutamatergic commissural-associational pyramidal cells (4), and, to a lesser extent, neurons of the contralateral entorhinal cortex (5).

Sprouting has been extensively studied by morphological methods, but relatively little is known regarding the molecular and biochemical events that underlie the sprouting response. Studies of protein synthesis in the dentate gyrus, as monitored by [^{3}H]leucine incorporation *in vivo*, show biphasic increases with peaks 6 and 12 days after ECL (2). Polyribosomes increase in the dendrites of dentate gyrus granule cells with a similar time course (6). A preliminary report using *in situ* hybridization showed increased β-tubulin and actin mRNAs in the molecular layer of the dentate gyrus 6 days after ECL (7). This evidence suggests that the synthesis of specific proteins is modulated during response to deafferentation.

Nonneuronal cell types are also involved in reactive synaptogenesis. Limited astroglial cell proliferation (30–40%) occurs in the hippocampus early after ECL (8). Ultrastructural studies of the dentate molecular layer show that throughout the time course of 2–14 days, astrocytes are the major phagocytic cells in initial responses. Their processes appear progressively to engulf both presynaptic terminals and preterminal axons (8, 9). Increased astrocyte reactivity, by immunoassays for glial fibrillary acidic protein (GFAP), is also typically observed during neurodegeneration—e.g., in Huntington disease, Joseph disease, and Alzheimer disease (10–12). However, other studies show that mRNA for GFAP can also be modulated by glucocorticoid treatment (13) or viral infection (Creuzfeldt–Jacob) (14) in the absence of noticeable neuronal loss. Astrocytes also produce neurotrophic factors that can influence the regenerative processes (15).

To approach the molecular events associated with the sprouting response, we examined alterations in the prevalence of various hippocampal mRNA species after ECL, using cell-free *in vitro* translation of a mRNA-dependent reticulocyte lysate system.

MATERIALS AND METHODS

Lesion. Young (3 months old) F344 male rats received complete bilateral electrolytic lesions to their entorhinal cortex ($n = 20$). A smaller number ($n = 7$) received unilateral lesions (16) for the neuroanatomical assessment of reactive synaptogenesis. Nine stereotaxic coordinates per side were lesioned with a 1-mA current of 45 sec duration. The electrode was positioned at a 10° angle from medial to lateral and the nose bar was lowered to −1.0. The first set of electrolytic lesion was done 3.3 mm lateral to midline and 0.2 mm posterior to interaural line. Lesions were made 2, 4, and 6 mm below the surface of the brain. The electrode was then repositioned at a point 1 mm lateral to the 3.3-mm point and was lowered successively to 2, 4, and 6 mm below the surface of the brain. Finally, three lesions were placed another 0.8 mm lateral and 1.0 mm anterior to the interaural line, and 2, 4, and 6 mm ventral from the brain surface. Pilot studies showed that the lesions targeted the medial and lateral entorhinal cortex and part of the parasubiculum. After a recovery period of 14 days, during which there was loss of weight, the animals were sacrificed and the brains were dissected rapidly. The hippocampi were frozen immediately on dry ice and stored at −80°C.

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Abbreviations: ECL, entorhinal cortex lesion; GFAP, glial fibrillary acidic protein.

†To whom reprint requests should be addressed at: McGill Center for Studies in Aging, 1650 Cedar Avenue, Montreal, PQ, H3G 1A4, Canada.

The efficacy of lesioning was verified histochemically by using acetylcholine esterase activity to assay for cholinergic sprouting (3, 17) and kainic acid binding to assay commissural-associational fiber sprouting (4). Paraffin-embedded sections were processed with modified Timm's staining (18) to highlight the degenerating fibers. Controls were sham operated and unoperated.

RNA Extraction. Frozen tissues were homogenized directly into a guanidinium isothiocyanate solution (5 M guanidium isothiocyanate/50 mM Tris·HCl/50 mM EDTA/4% 2-mercaptoethanol) and RNA was isolated by centrifuging the homogenate through a 5.7 M cesium chloride cushion (19).

***In Vitro* Translation.** The micrococcal nuclease-treated rabbit reticulocyte lysate (BRL) was used to translate 5 μg of total RNA from two sets each of control and lesioned hippocampi (pooled from 10 rats) with [^{35}S]methionine (15, 20). Morrisson *et al.* (21) showed that the relative translation of different mRNAs is unaffected by the presence of a large amount of rRNAs when total RNA is isolated by guanidinium extraction.

Two-Dimensional Protein Gel Electrophoresis. Equal amounts of [^{35}S]labeled *in vitro* translation products (100,000 trichloroacetic acid-precipitable cpm) were resolved by two-dimensional gel electrophoresis (13, 22) and visualized by fluorography (20). Each sample was run in duplicate in two separate experiments representing separate groups of 10 rats each.

Computer-Assisted Densitometric Scanning. After fluorography, gels were exposed to x-ray film for three different durations (3, 5, and 21 days) to evaluate possible nonlinear responses of the films. The autoradiograms were digitized (optical density scanner system; Technology Resources, Nashville, TN) and values were expressed as total optical density units (optical density × area of the signal). The confidence interval used to evaluate the statistical significance of changes after ECL for each gel spot of interest was established with reference to eight different spots on the autoradiogram that appeared unmodified by lesioning. From the corresponding optical density values for each spot, a regression analysis was performed. The amount of *in vitro* translation product for GFAP and the control spot c1 was directly assayed from punches of the gel containing the spots (Fig. 1). The gel punches were then solubilized and the radioactivity was determined by scintillation counting for comparison with autoradiogram values.

RNA Titration of GFAP. Solution hybridization and RNase protection assays for GFAP were done according to Lee and Costlow (23) using a mouse GFAP cDNA clone generously provided by Nicholas J. Cowan (New York University Medical Center). This 1.2-kilobase clone contains >50% of coding sequences. Briefly, RNA from lesioned and control total RNA were hybridized with a GFAP [^{32}P]-labeled complementary RNA probe in 10-fold excess and were subsequently digested with a mixture of RNases T1 and A that recognize and degrade unhybridized single-stranded RNAs. The duplex RNAs were acid precipitated and collected on GFC filters and the radioactivity was counted. The prevalence was estimated by regression analysis of the amount of acid-precipitable radioactivity versus the amount of total RNA present in the assays. Additional Northern blot analyses were done using a mouse α_1-tubulin cDNA probe (near full length; from Nicholas J. Cowan) and two rat β-tubulin cDNA probes encoding an early development specific tubulin isomer and a late development tubulin isomer (gift from Steven Farmer, Boston University Medical Center).

RESULTS

Rats were examined 14 days after ECL, the midpoint of maximum synaptogenesis (24, 25). Yield of total hippocampal RNA was similar in control (763 and 851 μg per g wet weight) and lesioned rats (832 and 731 μg per g wet weight) in pooled hippocampi from 10 rats each. The template activity of total RNA was assessed *in vitro*: incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable proteins was similar in both groups (control: mean, 20,430 cpm per μg of total RNA; lesioned animal mean, 20,900 cpm per μg of total RNA).

Of the 300 spots resolved per gel, we identified <10 select changes after ECL (Fig. 1). Spots labeled c1–c8 represent nonchanging peptides, while spots x1–x7 designate polypeptides that are consistently increased by ECL. Signals showing

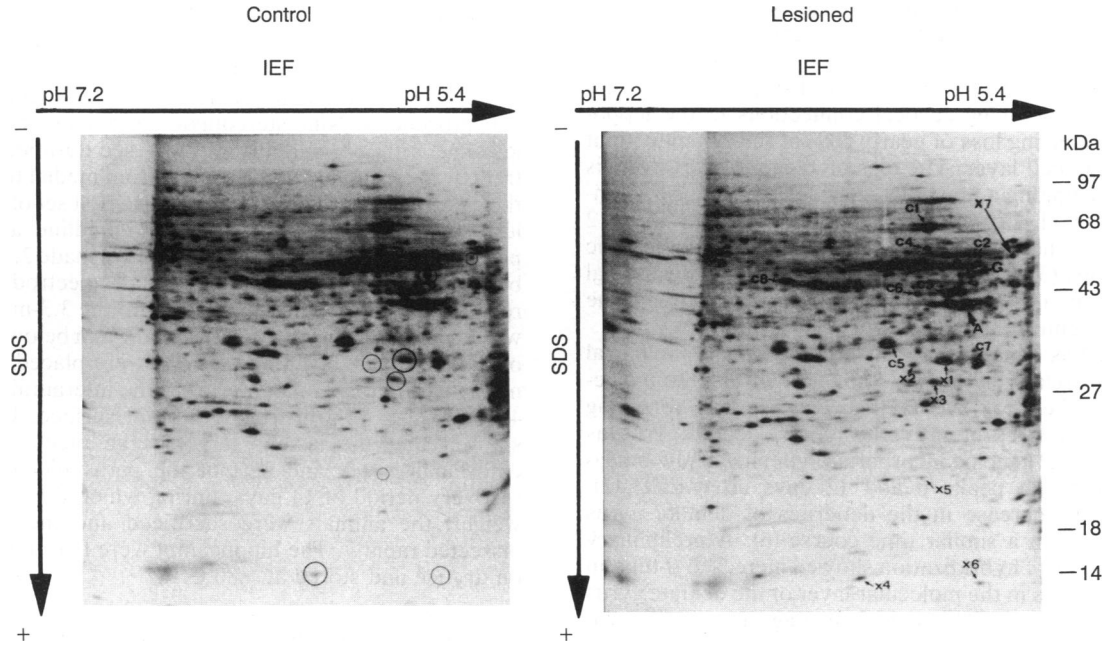

FIG. 1. Comparison of polypeptides synthesized *in vitro* by hippocampal total RNA isolated from control and entorhinal cortex lesioned rats (14 days postlesion). A, actin polypeptide; G, GFAP. Responsive unidentified polypeptides are labeled x1–x7, while unchanging references are labeled c1–c8. The two-dimensional gel fluorographs were obtained after 3 weeks of exposure. IEF, isoelectric focusing.

>1.5-fold increase of intensity in ECL vs. control indicate significant changes (Fig. 2). Fig. 2 shows that the signal ratio between the two groups remains linear over a range of 1–32,000 arbitrary optical density units. To validate the digitized autoradiographic values, the radioactivity (cpm) of two well-defined polypeptides (c1, a control; GFAP, a changer) was assessed directly in punches from the gel. Table 1 compares the results obtained by both direct counting and digitized autoradiography of the spots. The digitized and direct counting values did not differ. Analysis of the ratio of total optical density for selected spots shows that mRNA for GFAP (50 kDa, pI 5.5), but not the actin isotype complex, is altered by ECL at the 14-day time point (Fig. 3). The identification of the GFAP signal is based on the similar location of GFAP with respect to actin on two-dimensional gels of translated mRNA from human and rodent brain (10, 26) and from hybrid select translation experiments (13). We also performed RNA titrations of GFAP using a mouse GFAP complementary RNA probe. The hybridization protection assay (Fig. 4) shows a 2- to 3-fold change in GFAP mRNA prevalence after ECL, which confirmed the *in vitro* translation experiments.

Seven other unidentified polypeptides were increased by ≥2-fold after ECL (Fig. 3). The molecular mass of these polypeptides varied from 12 kDa up to 53 kDa; they were slightly acidic (Table 2). The region of the gel where the isotypes of tubulin are known to migrate contains a peptide (x7, 53 kDa) whose RNA increases 7-fold after ECL. Northern blot analysis of the control and lesion RNA using probes for α_1-tubulin and late and early development β-tubulin did not detect any alteration of the tubulin mRNA's prevalence 14 days after ECL (data not shown). This is consistent with a recent report by several of the present authors that ECL causes a transient increase in the α-tubulin mRNA at 6 days and a return to basal level at 14 days (27). Moreover, since we did not observe any increase in actin mRNA complex prevalence at 14 days postlesion, we assume that the increase reported by Phillips *et al.* (7) at 6 days also represents a transitory phenomenon like α-tubulin.

Table 1. Comparative analysis of digitized densitometric values and direct gel radioactivity counting of the selected polypeptides

	Ratio ECL/control	
	Direct quantitation of gels	Image analysis of autoradiograms
c1	1.03 ± 0.09	1.02 ± 0.19
GFAP	2.35 ± 0.30	2.15 ± 0.43

Each value represents the average ratio ± SEM of ECL versus control spots obtained from four individual measurements. c1 represents a control spot (see Fig. 1).

DISCUSSION

These studies show changes in the quantity of eight hippocampal mRNA sequences 14 days after ECL. Among the major changes, GFAP mRNA levels assessed by *in vitro* translation were increased after ECL.

GFAP is used extensively as an index of astroglial reactivity in several areas of the brain where it is increased by viral, chemical, mechanical, and electrical injuries. Similarly, GFAP increases in neurodegenerative diseases (10, 11). The 2-fold or more increase in GFAP mRNA prevalence after ECL greatly exceeded the 35% increase in the number of astrocytes in the denervated zone (16, 28). Similarly, a study on astrocytic hyperplasia by Manuelidis and Manuelidis (29) showed that the [^{3}H]thymidine incorporation only rarely labeled astroglial nuclei during the period when GFAP mRNA is increased after stab wounds.

The intracellular pathway for GFAP mRNA induction in the hippocampus by entorhinal deafferentation is unknown. But the fact that astrocytic processes can engulf both presynaptic terminals and preterminal axons during the critical phase of sprouting and reactive synaptogenesis (8, 24) sug-

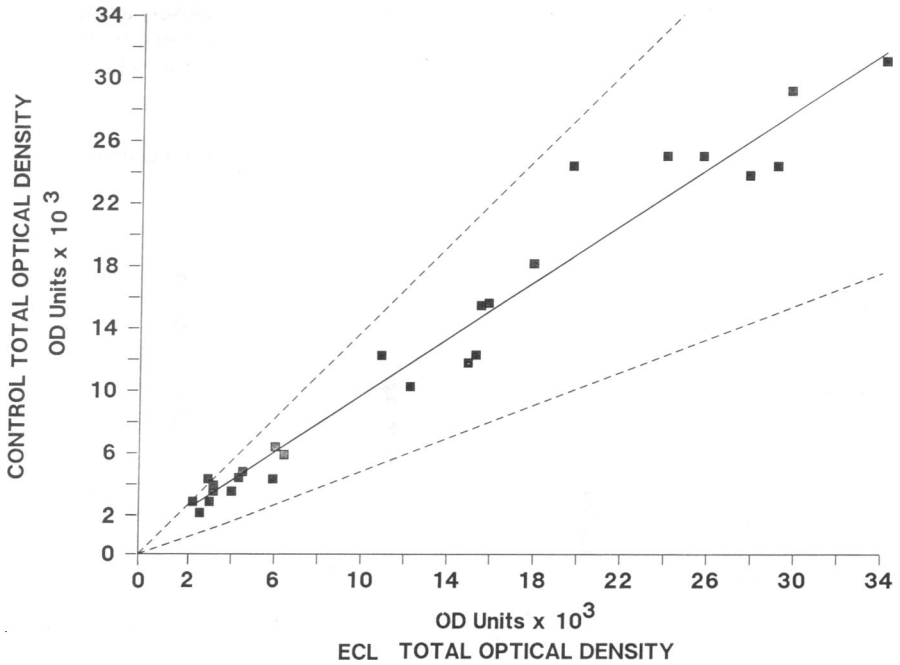

FIG. 2. Regression analysis of the optical density ratio of eight nonchanging spots obtained from four pairs of fluorographs. Each point represents the ratio of control versus lesion values obtained from that spot in one pair of fluorographs. To determine the variance of the nonchanging spots in ECL and control electrophoretograms, the values for eight nonchanging spots were plotted for each pair of the lesioned and control hippocampi and a regression analysis was performed. The slope, which determines the ratio between control and lesioned animals, is equal to 0.91 ± 0.17 (mean ± SD; $r^2 = 0.97$). This result indicates that the average intensity of the nonchanging spots does not differ between the ECL and control autoradiograms. A confidence interval that encompassed 3 SD from each side of the slope value (represented by dashed lines) was subsequently defined to ensure $P < 0.01$.

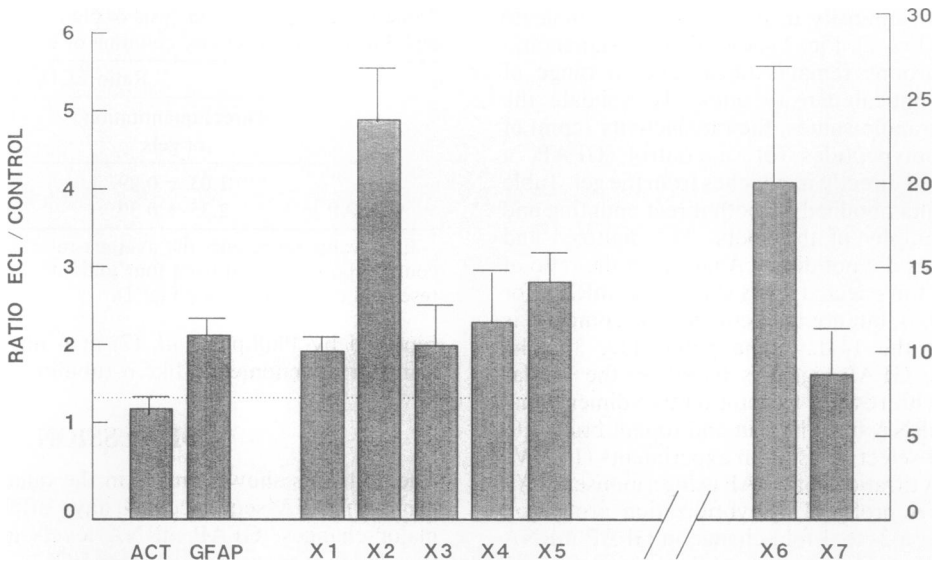

FIG. 3. Ratio of hippocampal RNA responses to entorhinal cortex deafferentation in the lesioned versus control rats. All the responses except actin significantly differ from the controls ($P < 0.01$). Dotted line represents the upper limit of the confidence interval. ACT, actin; x1–x7 represent unidentified changing polypeptides.

gests a direct relation between degenerating fibers and astroglial membranes. Such interactions could in turn modulate the gene expression of specific transcripts like GFAP mRNA. Interestingly, glucocorticoid administration, which retards reactive synaptogenesis (30), also decreases GFAP mRNA in the hippocampus of unlesioned rats (13). Taken together these data strongly support the concept of several roles for astrocytes during reactive synaptogenesis.

It is of interest to compare the effects of ECL in rats with histochemical changes seen in some patients with Alzheimer disease. As observed for rats after ECL, septal and commissural-associational fibers sprouted in the hippocampus during Alzheimer disease (4, 31, 32), presumably in response to neuron loss in layer II of the entorhinal cortex (33). Moreover, in patients with Alzheimer disease, the hippocampus presents a 2- to 3-fold increase in GFAP mRNA prevalence (P.C.M. and C.E.F., unpublished observations). However, no link can be implied between GFAP expression in Alzheimer disease and the local reactive synaptogenesis since, contrary to the rodent model, the hippocampus in Alzheimer disease patients has pyramidal neuron degeneration that might independently provoke astrocyte responses.

Three of the peptides (x4–x6) are in the small size and acidic pI range of polypeptide neuroregulators—namely, interleukin 1 (17–18 kDa), which stimulates reactive astrogliosis (34) and fibroblast growth factor (15 kDa) (35). Other small regulators include nerve growth factor (13.2 kDa), a neurotrophic factor closely associated with acetylcholinergic cell survival and recovery from transection (36, 37); endothelial cell growth factor, which promotes survival of endo-

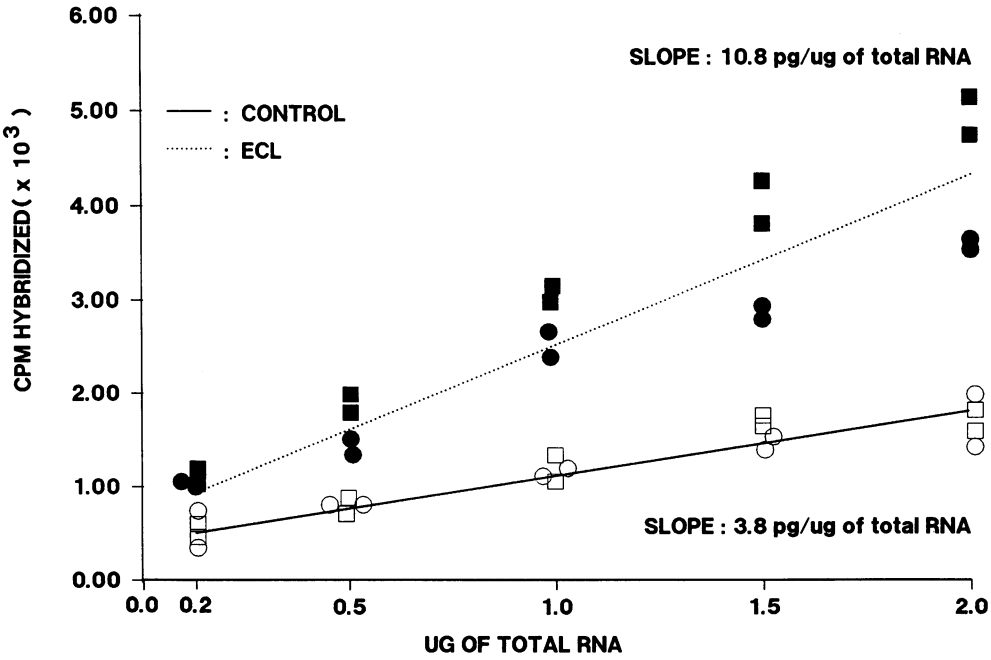

FIG. 4. GFAP mRNA titration assays of total RNA extracted from two sets of 10 control rats and two sets of 10 lesioned rats (14 days postlesion). Each assay was run in duplicate and the slope was determined by regression analysis. The difference between control and experimental rats is significant ($P < 0.001$).

Table 2. Molecular masses and isoelectric points of the unidentified changing polypeptides

Polypeptide	Molecular mass, Da	pI
x1	35,000	5.6
x2	33,000	5.7
x3	31,000	5.7
x4	13,000	6.1
x5	20,000	5.8
x6	12,000	5.6
x7	53,000	5.4

thelia *in vivo* (38); and brain-derived neurotrophic factor (12.3 kDa), which promotes survival of retina ganglion cells and sensory neurons (36). However, ECL in rat neonates did not induce any changes in nerve growth factor mRNA prevalence 10 days after the lesion (9). Previously, Nieto-Sampedro *et al.* (15) showed that ECL induces an increase in neurotrophic factor activity, which peaks ≈14 days postlesion. The unknown responding RNA sequences could be neural or glial, since total RNA was isolated from whole hippocampus. Because the unidentified changing polypeptides (x1–x7) are in the intermediate to abundant prevalence class, their RNA should be amenable to cloning. Meanwhile, these markers should be useful for characterizing molecular changes during reactive synaptogenesis.

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