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Early Maternal Separation increases NGF Expression in the Developing Rat Hippocampus

FRANCESCA CIRULLI,*† ALESSANDRA MICERA,‡
ENRICO ALLEVA* AND LUIGI ALOE‡*

*Section of Behavioral Pathophysiology, Laboratorio di Fisiopatologia di Organo e di Sistema, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161, Rome, Italy,
†Neurosciences Program, Stanford University School of Medicine, Stanford, CA 94305, USA, and
‡Institute of Neurobiology, Consiglio Nazionale delle Ricerche, Viale Marx 15, I-00137 , Rome, Italy

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CIRULLI, F., A. MICERA, E. ALLEVA AND L. ALOE. *Early maternal separation increases NGF expression in the developing rat hippocampus*. PHARMACOL BIOCHEM BEHAV **59**(4) 853–858, 1998.—Nerve Growth Factor (NGF) is a neurotrophin involved in growth and differentiation of central cholinergic neurons. In this study a maternal separation paradigm was used to test whether levels of NGF might be affected by brief manipulations of rat pups early during ontogeny. The expression of NGF mRNA was examined in 3-day-old rat pups following 45 min maternal separation using in situ hybridization. Early maternal separation in neonatal rats resulted in increased expression of NGF mRNA in the dentate gyrus and the hilus of the hippocampus. NGF protein levels measured (by means of a sensitive ELISA assay) in the whole hippocampus the day following the separation procedure did not differ in separated vs. nonseparated pups. These data indicate that brief manipulations performed early during development can affect hippocampal NGF expression. © 1998 Elsevier Science Inc.

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EARLY separation and loss constitutes a psychological risk factor in borderline personality disorder (4). Recent evidence suggest the possibility that changes in neurotrophic factor availability during specific developmental windows might be implicated in the pathogenesis of psychiatric disorders such as schizophrenia. Indeed, an association has been found between specific DNA variants of the Neutrotrophin-3 (NT-3) gene and severe forms of schizophrenia (15).

Early mother–infant separation in nonhuman primates has been extensively used as a means to induce experimental psychopathology, especially anaclitic depression (37). Following mother–infant separation a behavioral protest response ensues with changes in heart rate, body temperature, vocalizations, REM sleep, and cortisol (26). Cerebral monoamine activity is also increased in the infant monkey after it has been separated from the mother (7). Long-term effects of mother–infant separation have been assessed (16). Peer-reared animals showed increased levels of monoamine metabolites in their cerebrospinal fluid and increased cortisol concentrations. In rodents, a complex physiological response ensues following maternal separation. In the rat pup responses to maternal separation include changes in heart rate, respiratory rate, and hormone secretion (17,20). Similarly to primates, changes in central monoamines and their metabolites in response to maternal separation have been documented in guinea pigs (39). However, changes in neurotrophic factors expression, as a result of maternal separation, have not yet been reported.

The hippocampus is an extremely plastic structure and the site of synthesis of neurotrophic factors both during development and in the adult animal (12,13,24,38,45). Nerve growth factor (NGF) is the best-characterized neurotrophic factor, acting as a target-derived trophic factor for basal forebrain cholinergic neurons involved in learning and memory processes (5,42). NGF can affect neurochemical differentiation of neurons (3) and it can influence remodeling of axonal as well as dendritic arborization and thus modify CNS connectivity (32). Also, a regulatory

Requests for reprints should be addressed to Dr. Luigi Aloe, Institute of Neurobiology, Consiglio Nazionale delle Ricerche, Viale C. Marx 15, I-00137, Rome Italy.

role in apoptosis has been demonstrated (31). Interestingly, environmental enrichment has been shown to lead to increased NGF levels in the hippocampus of adult rats, and this was also correlated with a better performance in a Morris maze task (29).

At variance from other brain systems, the hippocampus, especially the dentate gyrus, undergoes major anatomical and physiological changes after birth in the rat. For example, almost all granule cells in the rat dentate gyrus are generated postnatally, and synaptogenesis and myelination occur in the hippocampal formation well into the fourth postnatal week (8,18). Because of these characteristics, this brain structure is extremely sensitive to changes in trophic factor availability occurring early during development that might result in the long-term effects seen in the handled animals. We thus sought to test whether changes in NGF expression might be detected in the hippocampal region at the end of a brief manipulation (45-min maternal separation) in newborn rats.

METHOD

Animals

The subjects used in these experiments were the offspring of male and female Sprague–Dawley rats (Charles River Laboratories; Calco, Italy) kept under standard laboratory conditions (temperature 21 ± 1°C, relative humidity 60 ± 10%, lights on from 0930 to 2130 h). Seven different litters were used. All litters were culled to eight pups the day following birth (day of birth = day 0). In the in situ hybridization study, on postnatal day 3, half of the pups in the litter (three litters used) were removed, placed in a plastic container and kept in an incubator at 32°C for 45 min and sacrificed at the end of this time period. The remaining pups were sacrificed immediately. This provided a control for any possible litter effects. Four litters were used for protein level determination. To detect changes in hippocampal NGF protein content following maternal separation, on postnatal day 2, half of the pups in the litters (four litters were used) were removed, placed in a plastic container, and kept in an incubator at 32°C for 45 min. At the end of this time period the offspring were returned to the mother. All pups were sacrificed on PND 3, their brains dissected, and the hippocampus isolated. Animal experiments were conducted according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) as implemented in Italy by the Legislative Decree 116/1992.

In Situ Hybridization

Brains were dissected and fresh frozen in liquid freon. Coronal brain sections (15 μm thick) were cut on a cryostat at −15°C. The sections were thawed onto poly-L-lysine (100 mg/ml; Sigma, Italia)-treated glass slides and then frozen. Before hybridization, the sections were fixed in 4% paraformaldehyde (Merck/BDH, Germany). For detection of NGF mRNA an antisense (45)-mer oligonucleotide complementary to a region of the NGF mRNA was used. The sequence of the oligonucleotide was as follows: 5′-TCGATGCCCCGGCACCCACTC-TCAACAG-GATTGGAGGCTCGGCAC-3′. The oligonucleotide probes were labeled at the 3′ end with alpha-^{35}S-dATP (DuPont, Italia) using terminal deoxynucleotidyl transferase (Boehringer–Mannheim, Germany). The labeled probe was purified from unincorporated nucleotides through a Sephadex G50 (Sigma–Aldrich, Italia) column and then DTT (Sigma–Aldrich, Italia) was added to a final concentration of 10 mM. The specific activity of the oligonucleotides ranged from 1–4 × 10^6 cpm/ng. Hybridization was performed as follows. The sections were first air dried and prehybridized for 1 h at room temperature in a buffer containing 50% formamide (Fluka, Italia), 5 × SSC (1 × SSC, is 0.15 M NaCl, 0.015 sodium citrate pH 7.0), 10 × Denhardt's solution, 0.1% SDS (Sigma–Aldrich, Italia), 50 mM sodium phosphate buffer (pH 7.0), 200 mg/ml heparin (Sigma–Aldrich, Italia), 250 mg/ml heat denatured salmon sperm DNA (Sigma–Aldrich, Italia) and 20 mM β-mercaptoethanol (Fluka, Italia). Subsequently, the buffer was discarded and substituted with 250 ml of fresh hybridization buffer enriched with 10% dextran sulphate (Pharmacia Biotech, Italia) and the ^{35}S-labeled probe (5 × 10^5 cpm) was homogeneously distributed over the sections by putting a piece of parafilm cut to size on top of the glass slides. In control experiments probes in the mRNA-sense orientation were used (5 × 10^5 cpm per section). The slides were placed in a humid chamber and incubated overnight at 42°C. Following hybridization the sections were rinsed six times in 2 × SSC + 0.1% SDS at room temperature for 1 h and washed overnight in 1 × SSC at 42°C. All solutions contained 20 mM β-mercaptoethanol. Finally, the slides were rinsed in distilled water, dehydrated in alcohol, and air dried. Sections were later dipped in photographic emulsion (Ilford K-5, Ilford, UK) and left at 4°C for a period of time about 3 weeks before being developed using Kodak D19 developer at 17°C for 6 min and fixed with Unifix (Kodak, USA) for 6 min. The tissue was counterstained with toluidine blue (0.1%) before analysis. The sections were examined in a Zeiss Axiophot (Zeiss, Germany) microscope equipped with dark- and bright-field condensers and photographed with Kodak Ektachrome color films.

In Situ Hybridization Data Analysis

For quantitation of NGF mRNA expression in brain sections, positive cells were counted in two hippocampal regions: the dentate gyrus and the hilus. The dentate gyrus was selected because cell birth as well as synaptogenesis and myelination occur in this area well into the fourth postnatal week and it is intimately connected with the hilus (8,18). All sections were hybridized under identical conditions and developed at the same time. Quantitation of grain clusters was done under dark-field illumination at 20× magnification. An average of five sections from level A 26 onward (35) were chosen for quantitation of granule-bearing cells in the two regions chosen for each maternally separated (MS) or nonseparated (NS) subject (n = 3 in each group). Data from sections hybridized with sense probes were used as background, and the resulting values subtracted from the original measurements to obtain the final values. All labeled cells showing a grain density at least three times higher than background were considered as positive cells. The number of positive cells per subject were averaged across sections as means ± SEM of all cells measured per region. Statistical differences between mean number of positive cells of the MS and NS groups were assessed by parametric t-tests.

NGF Determination

The levels of NGF were measured by a highly sensitive two-site immunoenzymatic assay that recognizes both human and murine NGF as previously described (41). The sensitivity of this assay is 5 pg/ml. Briefly, polystyrene 96-well microtubes immunoplates (Nunc, Denmark) were coated with affinity purified polyclonal goat anti-NGF antibody diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA) for evaluation of the nonspecific signal. After an overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05 M carbonate buffer pH 9.5 + 1% BSA), plates were

washed with Tris-HC1 pH 7.4 50 mM, NaC1 200 mM, 0.5% gelatine, 0.1% Triton X-100). After extensive washing of the plates the samples and the NGF standard solutions were diluted with sample buffer 0.1% Triton X-100 (100 mM Tris-HCl pH 7.2, 400 mM Nacl, 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA, and 0.5% gelatin; Sigma–Aldrich, Italia), distributed into the wells and left at room temperature overnight. The plates were then washed and incubated with 4 mU/well anti-β-NGF-galactosidase (Boehringer–Mannheim, Germany) for 2 h at 37°C and, after further washing, 100 μl of substrate solution (4 mg of chlorophenol red (Boehringer–Mannheim, Germany)/ml substrate buffer: 100 mM HEPES, 150 mM NaC1, 2 mM $MgC1_2$, 0.1% sodium azide, and 1% BSA) were added to each well. After an incubation of 2 h at 37°C the optical density was measured at 570 nm using an ELISA reader (Dynatech MR 5000, PBI International), and the values of standards and samples were corrected by subtraction of the background value due to nonspecific binding. Data are represented as pg/ml or pg/gr wet weight and all assays were performed in triplicate.

RESULTS

As shown in Fig. 1A–D, a greater number of NGF mRNA-positive cells were found both in the hilus and the dentate gyrus of maternally separated animals compared to the nonseparated littermates. Figure 1E shows the results of the data quantitation, each data point representing the average number of positive cells found in each of the two hippocampal regions examined for each experimental subject. The number of NGF mRNA positive cells was found to be higher in both the hilus and the dentate gyrus of maternally separated subjects compared to the nonseparated ones, $t(4) = 3.19$ and $t(4) = 2.77$, $p < 0.05$, respectively, for hilus and dentate gyrus. A significant difference in basal NGF mRNA expression was found in dentate gyrus and hilus of nonseparated subjects, $t(4) = 3.0$, $p < 0.05$. This difference was no longer evident following maternal separation.

NGF protein levels were determined by means of an ELISA assay performed on the whole hippocampus. This assay is able to detect levels of NGF as low as 5 pg/g wet weight. Unlike NGF mRNA, NGF protein levels determined the day following the maternal separation episode were not significantly different from those measured in nonseparated subjects (Fig. 1F). A tendency for NGF protein levels to decrease was evident, although it did not reach statistical significance.

DISCUSSION

In this study a "maternal separation" model has been used to test whether the levels of NGF, a neurotrophin involved both in growth and differentiation of central cholinergic neurons as well as in neuronal microplasticity, apoptosis and learning, and memory processes, could be modified by a short-term maternal separation (3,5,31,32,42).

The present study shows that early maternal separation in neonatal rats results in increased expression of NGF mRNA in the dentate gyrus and the hilus, while protein levels for this factor are not modified the day following the manipulation. Cell death also appears to be increased in dentate gyrus granule cells (data not shown). This latter observation is consistent with the findings reported by other authors who found that stress is associated with neuronal loss within the hippocampal structure (33). The reason why a brief separation from the mother is able to increase NGF mRNA levels, while leaving unchanged or even decreasing the levels of the protein is, at the present time, unknown. One possible explanation is that, while the in situ hybridization data and thus the morphometric examination were restricted to a well-localized hippocampal area, the ELISA measured the amount of NGF protein content in the whole hippocampal formation possibly underestimating local changes in NGF. Furthermore, NGF turnover might be modified following maternal separation, and more protein might be needed by the neurons than is expressed, especially at an early developmental stage. Recent data show that NGF treatment in PC 12 cells results in increased endocytosis of ligand and receptor, a process that might ultimately lower NGF levels extracellularly (14).

As for the mechanism(s) involved in the regulation of NGF expression, an increasing body of evidence suggests a role for hormones and cytokines in modulating NGF expression (22). In the adult rat, limbic seizures as well as glutamate increase NGF mRNA in the rat hippocampus and cortex (10,11,46). Recent observations also support the regulation of NGF expression by behavioral arousal. Spillantini et al. (36) showed that intermale fighting leads to a marked increase in NGF mRNA in the adult mouse hypothalamus. Changes in central monoamines and their metabolites in response to maternal separation have been documented in monkeys and guinea pigs (7,39). Whether any or a combination of these mediators is responsible for the increase in NGF expression subsequent to maternal separation still needs to be established.

The increase in NGF expression that follows a brief maternal separation could modify brain plasticity to redirect brain growth, following external stimulation. During development, NGF affects growth and biochemical differentiation of basal forebrain cholinergic neurons (27,28,42). Repeated intraventricular administration of NGF results in a selective increase in ChAT activity in both the basal forebrain and its cholinergic projection areas in postnatal and adult rats (19,27,28). Conversely, intraventricular administration of NGF antibodies reduces ChAT activity in adult basal forebrain and its projections (40). Moreover, behavioral reactivity under cholinergic control can be modified by NGF treatment. In the neonatal mouse, intracerebroventricular administration of NGF caused a precocious appearance of scopolamine-induced hyperactivity (6).

Based on the data reported here we hypothesize that the hippocampus, through the release and its own ability to respond to this neurotrophin, might be involved in several of the events that underlie the long-term changes characterizing animals manipulated in infancy (1,2,21,23,25,43). Indeed early experiences speed up the emergence of exploration as well as the ontogeny of place learning and increase long-term potentiation (LTP) in young rats (25,30,44). Because of the role played by NGF in the development of the CNS, and in particular on cholinergic functioning, changes in the levels of this neurotrophil during critical developmental windows might affect cell differentiation or connectivity, ultimately leading to long-term changes in hippocampal functioning. Further experiments are currently in progress to assess whether the changes in NGF expression here reported represent a transient phenomenon or whether they are long lasting.

Early changes in cerebral excitability or synaptic density have been hypothesized to underlie some psychiatric disorder such as schizophrenia (34). It is interesting to notice that growth and neurologic development in premature human infants are improved by active tactile stimulation (9). Our data suggest that psychiatric disorders, such as schizophrenia, may likely result from the cooperative effect of neurobiological (NGF-controlled cholinergic development) and behavioral end points.

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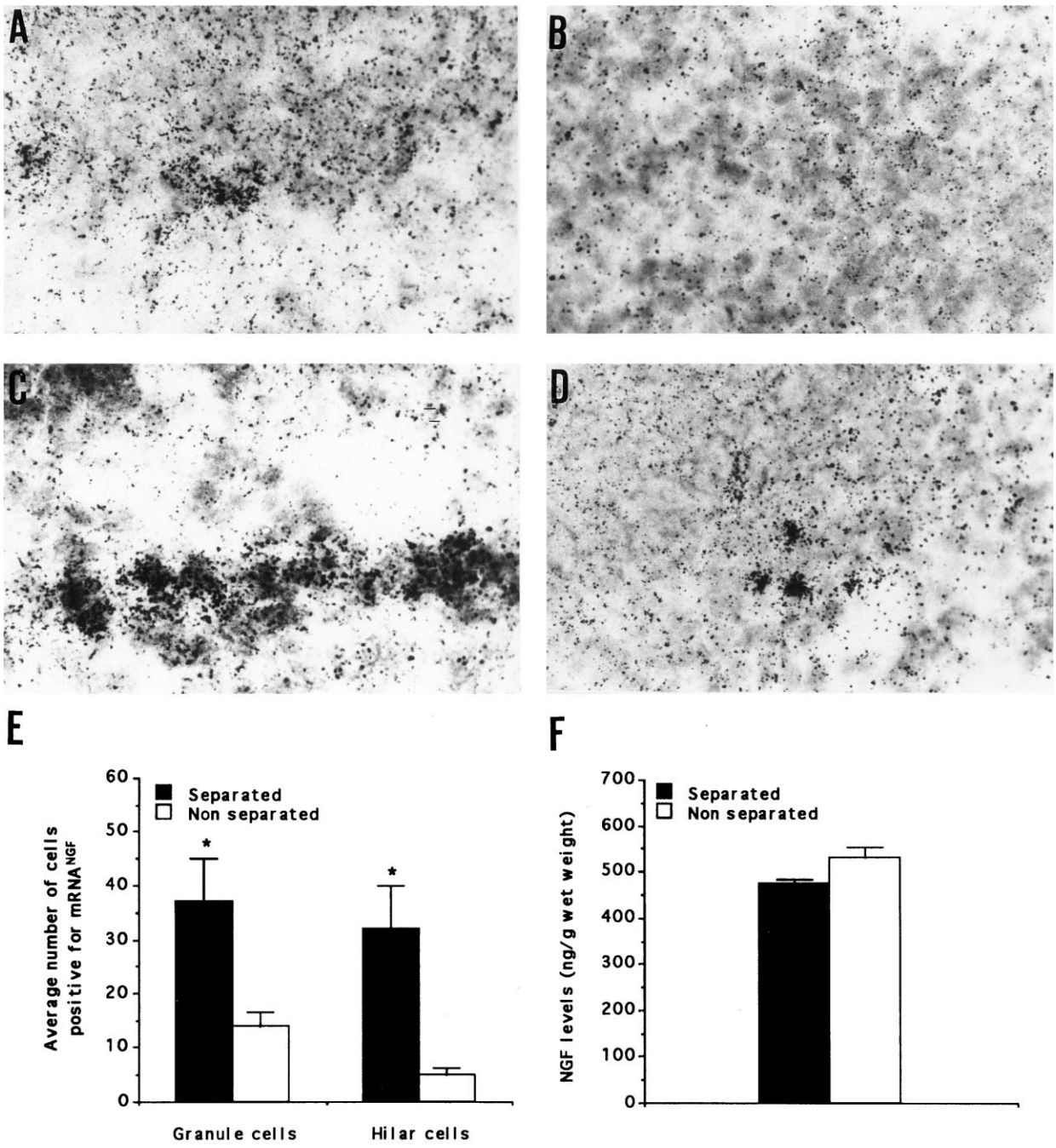

FIG. 1. In situ hybridization for $mRNA^{NGF}$ in the rat hippocampus and protein levels determined the day following the separation procedure. In situ hybridization for $mRNA^{NGF}$ in the rat dentate gyrus (A) and in the hilus (B) of control subjects and (C) and (D)following 45-min maternal separation on postnatal day 3, respectively, in the same brain regions. A greater number of $mRNA^{NGF}$ positive cells were found both in the dentate gyrus (C) and hilus (D) of maternally separated animals compared to the nonseparated ones (A) and (B). (E) Results of the data quantitation. Sections from the separated or nonseparated (control) subjects were hybridized to an NGF antisense riboprobe. Each data point represents the average number of positive cells found in each of the two hippocampal regions examined for each experimental subject. The mean number of positive cells in the hilus and dentate gyrus of separated ($n = 3$) and nonseparated subjects ($n = 3$) was compared. There were significantly more cells positive for $mRNA^{NGF}$ in both the hilus and the dentate gyrus of maternally separated subjects ($p < 0.05$). A significant difference in basal $mRNA^{NGF}$ expression was found in dentate gyrus and hilus of nonseparated subjects ($p < 0.05$). This difference was no longer evident following maternal separation. (F) NGF protein levels were determined by means of an ELISA assay performed on whole hippocampal tissue. The experimental subjects were separated on postnatal day 2 for 45 min and their hippocampus dissected on the following day. NGF protein levels determined the day following the maternal separation episode were not significantly different from those measured in non-separated subjects.

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