Reduced Nerve Growth Factor in Rett Syndrome Postmortem Brain Tissue

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Abstract. To determine whether reduced nerve growth factor (NGF) and/or its high affinity receptor, trkA, play a role in the pathophysiology of Rett syndrome (RS), we used immunohistochemistry in paraffin-embedded human autopsy brain tissue to quantify NGF and trkA levels within the frontal cortex of 9 RS females and 10 female controls of similar age. The results showed a significant reduction of NGF expression in RS patients (p < 0.001). Specifically, all RS brains exhibited NGF levels at or below the minimum level observed in controls. In 3 RS brains there was no NGF detected. TrkA expression was also reduced in the RS group (p = 0.035). Interestingly, the expression of NGF in the RS group was significantly related to the presence of cortical astrogliosis (r = 0.91) as indicated by immunostaining for glial fibrillary acidic protein (GFAP). This suggests that while the signals for NGF production during injury remain intact, the critical developmental signals required for early NGF production are impaired.

Key Words: Astrogliosis; GFAP; NGF; Rett Syndrome; trkA.

INTRODUCTION

Rett syndrome is a childhood neurodevelopmental disorder in girls characterized by a severely disabling mental and physical condition. It occurs in approximately 1:10,000 female newborns and represents up to 30% of progressive encephalopathies of unknown etiology (1). Development proceeds normally until approximately 6 months to 3 yr of age with the majority of cases developing before 18 months (2). Usually autistic behavior is noticed first, followed by loss of purposeful hand movements, inability to communicate, spasticity, and seizures (2–4). The condition eventually stabilizes; however, impaired ambulation and communication persist throughout life resulting in the need for long-term institutionalization (2).

Pathological examination reveals a generalized reduction in brain weight with a significant reduction of basal and apical dendrites within layers 3 and 5 of the cerebral cortex (5–7). Reduced dendritic arborization and synapse formation is believed to be due to a developmental failure of the second critical phase of synaptogenesis, which normally occurs between 2 and 18 months of age (5, 8). However, the reason for this failure in development is currently unknown.

Studies have shown that NGF is responsible for the later developmental stages of dendritic outgrowth and is believed to be vital during the final stages of synaptogenesis (9, 10). NGF and its receptor trkA are predominantly expressed within the pyramidal cells in layers III and V of the cerebral cortex (9, 11); the same layers most severely affected in Rett syndrome. Since neurons in this area become NGF dependent during the final stages of synaptogenesis, it is our hypothesis that patients who develop Rett syndrome lack an adequate expression of NGF, especially critical during this time period.

In this investigation we used immunohistochemistry in paraffin-embedded human autopsy brain tissue and digital image analysis to quantify NGF and trkA expression within the frontal cortex from 9 patients with RS and compared them to 10 controls of similar age. The frontal cortex was chosen because it is one of the areas most consistently affected in RS brains (5–7). TrkA was quantified in these patients to further elucidate receptor/ligand dynamics involved in NGF activity. In addition, immunostaining for GFAP was used to determine the presence of cortical astrogliosis, a potential source of injury-induced NGF production that may have occurred after the onset of RS.

MATERIALS AND METHODS

Subjects

Postmortem brain samples from 9 RS females, ages 7–41 yr, were selected and kindly donated by the Harvard Brain Tissue Resource Center. Ten female controls, ages 3–49 yr, were selected to age match the RS tissue and donated by the Department of Pathology at Tulane Medical Center and by Dr. Glenda Henderson at British Columbia Children’s Hospital in Vancouver. The diagnosis of RS in all subjects was made by experienced clinicians based upon the classic criteria for RS (3). The most common cause of death for the RS group was either cardio-pulmonary abnormalities or seizures, with a postmortem interval (PMI) range from 2.5–83.0 h. For the control group, the most common cause of death was cardiac arrest with a PMI range from 10.5–44 h. In either case, the possibility that PMI might influence NGF, trkA, or GFAP expression was carefully considered. The control and RS groups did not differ significantly with respect to age (p = 0.377) or PMI (p = 0.926). All tissue samples were excised from the frontal lobe of each subject, fixed in standard 10% formalin, and paraffin-embedded prior to sectioning. Five-micron adjacent coronal sections were...
processed for immunohistochemical staining of NGF, trkA, and GFAP.

**Immunohistochemistry for NGF and TrkA**

Paraffin slides were dried at 60°C overnight. Sections were deparaffinized by successive washings in xylene (5 min) twice, 100% ETOH (1 min), and 95% ETOH (1 min). Endogenous peroxidase activity was blocked using 0.6% H₂O₂/MeOH and incubated for 30 min at room temperature (RT). After thorough washes in deionized water, the slides were placed in Antigen Retrieval Citra Solution 10X, HK086-9K (AR) (Biogenex) at 1:9 (1 part AR to 9 parts deionized water) and brought to a boil in the microwave for 10 min. Sections were again rinsed thoroughly and blocked in 20% goat serum/PBS with Tween 20 (Sigma) for 30 min at RT. Polyclonal rabbit anti-human NGF and trkA (RDI, Flanders, NJ) were used as primary antibodies diluted 1:1,000 in 10% goat serum. One section from each specimen received a competition control solution in which a competition protein (RDI) was added to the primary antibody solution equal to 10X the antibody concentration by weight (1:100). In another section from each specimen, the primary antibody solution was replaced with PBS. Slides were incubated overnight at RT. After washing extensively in PBS with Tween 20, sections were incubated with the secondary antibody, biotinylated anti-rabbit immunoglobulin (Biogenex, Supersensitive B-S-A Immunodetection System) for 30 min at RT. Slides were again washed in PBS with Tween 20 before incubation in peroxidase-conjugated streptavidin (Biogenex). Reactions were developed with diaminobenzidine (DAB) and counterstained in hematoxylin. Sections were rinsed thoroughly and dehydrated in successive washings of 95% ETOH (2 min), 100% ETOH (2 min), and xylene (2 min) twice and prepared for non-aqueous mount and cover slip.

NGF and trkA-positive cells were not detected in sections in which excess control peptide was incubated with the primary antibody and resembled those in which the primary antibody solution was replaced with PBS. NGF-positive cells have been found in several studies to co-express trkA receptor (12–14). This would explain the similar staining pattern of NGF and trkA observed in this study. Both proteins were generally localized to the cytoplasm within the cell body and neurite extensions, although occasionally nuclear GF immunoreactivity was also noted. This observation is supported by previous studies in which NGF was found within the nucleoplasm (15, 16), providing additional evidence for the specificity of our NGF antibody.

**Immunohistochemistry for GFAP**

Mouse monoclonal anti-human antibody against GFAP was diluted 1:100 according to commercial protocols (DAKO Carpinteria, CA). This antibody has been well characterized by the Department of Pathology at Tulane University Medical School and used in routine staining of autopsy material. After incubation in the primary antiserum for 2 h at RT, sections were extensively washed and then incubated for 1 h at RT with the appropriate secondary antibodies diluted 1:100. Sections were again washed extensively before incubation in avidin-biotin horseradish peroxidase complex (DAKO). Reactions were developed in DAB.

**Quantification of NGF and TrkA**

We used Image-Pro Plus (Media Cybernetics) software to digitally quantify our data. With this system we were able to quantify the mean optical density (OD) of our DAB stain that reflects the amount of labeled protein. Optical density analysis determines the amount of labeled protein in a tissue section by measuring the amount of light it transmits. The software uses the following standard light transmission formula for calculating OD from intensity: OPTICAL DENSITY \( (x, y) = \log \left( \frac{\text{INTENSITY}(x, y) - \text{BLACK}}{\text{INCIDENT} - \text{BLACK}} \right) \)

Where: INTENSITY \( (x, y) \) is the intensity at pixel \( (x, y) \), BLACK is the intensity generated when no light passes through the material, and INCIDENT is the intensity of the incident light. During OD measurements, the Color Segmentation command is in force. This is used to separate labeled protein from the background based upon its color characteristics. In this investigation the DAB color was selected by the investigator, after which the computer identified the entire area within a given 40X field that contained the color. Next the computer calculated the average OD of the DAB stain within the field. Each calculation was obtained blindly and involved the analysis of from 75–150 cells per sampled field. In each section that was analyzed, 4 to 5 random samples throughout the cortex were measured. Density values from the sampled area were averaged for each section. Mean densities for each specimen were derived from the analysis of 2 sections. This technique proved to be reproducible in consecutive sections from the same specimen during separate trial periods (Table 1). Therefore, a single section from 1 specimen with a previously calculated OD value was used in each trial as a positive control. In addition, the ability to detect subtle variations of NGF expression was demonstrated using a series of increasing concentrations of primary antibody. Using this technique we were able to detect OD values corresponding to concentration changes along a saturation curve (Fig. 1). With regard to the Supersensitive B-S-A Immunodetection System (Biogenex), used according to the manufacturer’s instructions, the signal follows a linear pattern of amplification.

**Quantification of Astrogliaosis**

Astrogliosis was evaluated using immunohistochemistry for GFAP. A more traditional semiquantitative approach was used...
as described previously (17). With this approach, both stain intensity and astrocyte morphology could be closely examined to determine astrocyte activity. Specimens were scored from 0–3 as either negative (0), normal (1), mild (2), or severe (3), where: 0 = no difference in immunohistochemical staining from negative controls, 1 = thin glial processes dispersed among a histologically unremarkable neuropil, 2 = glial cells with well-defined processes, some of which outline small vessels, and 3 = glial cells with abundant processes within a histologically perturbed neuropil. All assessments were made blindly and independently by at least 3 authors. Scoring was based exclusively on labeled astrocytes within the cortical layer from each specimen.

Statistical Analysis

Unless otherwise noted, all between-group differences were tested using analysis of variance (ANOVA).

RESULTS

Data for control and RS groups are shown in Table 2. Measures of OD for NGF showed that NGF expression was significantly lower in RS patients compared with controls (p < 0.001) (Fig. 2). The mean NGF OD for RS patients was 15.33 (SD = 12.50) versus a mean of 31.80 (SD = 2.02) for controls. The data in Table 2 show that all RS patients exhibited NGF expression below the mean for controls. The maximum score among RS patients was 15.00, p = 0.935).

RS patients also exhibited reduced trkA expression versus controls (p = 0.035) (Fig. 3). Mean trkA OD was 20.22 (SD = 2.73) for RS patients and 22.50 (SD = 1.51) for controls. TrkA expression was not significantly related to PMI (r = 0.154, p = 0.542).

When GFAP labeling was examined, normal scores of 1 were found in all controls except the oldest subject, aged 49 yr, who exhibited mild astrogliosis (GFAP score of 2). In contrast, none of the RS patients had a normal GFAP score of 1. In 4 of the 9 RS patients no GFAP was detected (GFAP score of 0), while increased levels (GFAP scores of 2 or 3) were seen in the remaining 5 patients. Although the mean GFAP scores for the 2 groups were similar (1.11 for controls and 1.33 for RS), this between-group difference in astrogliosis was statistically significant when evaluated using chi-square (χ²(3) = 15.00, p < 0.001). These data suggest that there are 2 subgroups of RS patients, each of which exhibits abnormal GFAP values. Table 2 reveals that GFAP scores in

### Table 2

<table>
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<tr>
<th>Subject</th>
<th>Age (yr)/Sex</th>
<th>NGF (OD)</th>
<th>trkA (OD)</th>
<th>GFAP</th>
<th>PMI (Hr)</th>
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<td>1</td>
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<td>14</td>
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<td>21</td>
<td>28</td>
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Abbreviations: NGF, nerve growth factor; OD, optical density; GFAP, glial fibrillary acidic protein; PMI, postmortem interval; *, data unavailable.

Fig. 2. Measures of optical density (OD) for NGF showed that NGF expression was significantly lower in Rett patients \( (n = 9) \) compared with controls \( (n = 10) \) \( (p < 0.001) \). Each data point represents mean OD values. Horizontal bars: ±SE.

Fig. 3. Measures of optical density (OD) for trkA showed that trkA expression was significantly lower in Rett patients \( (n = 9) \) compared with controls \( (n = 10) \) \( (p = 0.035) \). Each data point represents mean OD values. Horizontal bars: ±SE.

RS patients are outside the normal range observed in all similarly aged controls. A significant direct relationship between GFAP and NGF expression was found for both the RS group \( (r = 0.91, p = 0.001) \) and controls \( (r = 0.679, p = 0.044) \) (Fig. 4). This indicates that increased NGF expression in both groups is associated with the presence of astrogliosis. GFAP was not significantly related to PMI \( (r = 0.032, p = 0.902) \).

Figure 5 demonstrates the relationship between NGF and GFAP expression in the control group (row 1) and RS group (rows 2 & 3). The first row shows a specimen from a control with labeled NGF (Fig. 5A) and GFAP (Fig. 5B), both of which represent normal stain intensity and morphology. The middle row shows a specimen from a Rett patient with labeled NGF (Fig. 5C) and GFAP (Fig. 5D). Although demonstrating nearly the same NGF OD as the control specimen (Fig. 5A) \( (30 \text{ vs } 33) \), the RS specimen reveals significant cortical astrogliosis (Fig. 5D). The bottom row shows a specimen from an RS subject negative for both NGF (Fig. 5E) and cortical GFAP (Fig. 5F). Like this specimen, all RS specimens with little or no NGF (i.e. OD \( = 0\text{–}15 \)) showed complete cortical sparing of GFAP labeling despite its presence in the white matter and subpial layer (not shown). This, in addition to positive cortical trkA labeling, indicates that the cortex was immunoreactive and that the stain for GFAP was effective but selectively absent in the cortex. The significance of this complete lack of cortical stain and whether it reflects some aspect of RS pathology is uncertain.

DISCUSSION

In the RS group, mean NGF OD values measured approximately 50% of controls with a range between 0 to the low end of normal. These results support a previous study in which reduced NGF levels were found in the cerebrospinal fluid (CSF) in patients with RS (18). In a subsequent study, normal serum NGF levels were detected in RS patients (19), which suggests that reduced NGF in RS is restricted to the central nervous system.

In the present investigation it was necessary to differentiate between the NGF produced in response to normal developmental signals and the NGF produced in response to astrogliosis. Classically, NGF is believed to be released by target cells and retrogradely transported to the cell body within receptive neurons (20, 21). Our hypothesis states that RS may be a consequence of inadequate NGF activity during a critical period of neuronal development. The inability to produce adequate levels at this time may explain the marked reduction of dendritic arborization...
Fig. 5. Photomicrographs demonstrating the relationship between NGF and glial fibrillary acidic protein (GFAP) expression in the control group (row 1) and in the Rett syndrome (RS) group (rows 2 and 3). The first row shows the cortex from a control subject. In (A), pyramidal neurons (n) are stained with DAB for NGF and in (B) astrocytes (a) are stained for GFAP. Both images represent normal intensity and morphology. The middle row shows an RS cortex labeled for NGF (C) and GFAP (D). Although having nearly the same NGF OD as the control (30 vs 33), the Rett cortex reveals significant cortical astrogliosis. The bottom row shows an RS specimen negative for both cortical NGF (E) and GFAP (F). In the last image (F), GFAP stain was present in the white matter (not shown) indicating that the stain was effective but selectively absent in the cortex. NGF and GFAP immunohistochemistry: hematoxylin counterstain, magnification ×400.

and number of dendritic spines of neurons in layers 3 and 5 of the cerebral cortex. The existence of astrogliosis in RS autopsy tissue may have been related to events that occurred shortly prior to death. However, since RS patients are prone to frequent seizure activity, they may be especially susceptible to reactive astrogliosis after the onset of symptoms. The fact that injury signals, such as those produced by frequent seizure activity, increase NGF expression (22–24) suggests the possibility of an alternative mechanism for NGF production in these patients. Furthermore, this may also explain the mild improvement of clinical symptoms observed in some RS patients during the plateau phase of the disease (25, 26).

Although several studies conclude that injury-induced NGF is produced by reactive astrocytes and microglia (14, 15, 23, 24) the source in these patients has yet to be determined. Upon close anatomical examination, however, reactive astrocytes within RS sections were found to coincide with NGF-positive areas in adjacent sections, indicating a close relationship between astrogliosis and NGF production.

Another interesting observation was that 1 RS subject had a mean NGF OD of 15 with no evidence of GFAP staining. This suggests that RS patients may have the potential to produce at least some NGF without the influence of astrogliosis. Such a phenomenon offers support...
for the X-linked model of RS with lethality in males (26–28). Studies suggest that the expression of the RS gene is influenced by the X-chromosome inactivation (XCI) pattern (29–31). A skewed XCI pattern in which the majority of cells express the normal region of the X chromosome (Xq28) is hypothesized to be responsible for asymptomatic carriers in mothers of RS girls (32, 33). Alternatively, a random or less favorably skewed distribution pattern is thought to allow the RS gene to be expressed more prominently resulting in clinical symptoms (32). The fact that some RS patients are able to produce some NGF independent of astrogliosis indicates that the expression of the RS gene varies among patients. One theory suggests that unequal XCI distributions explain the different clinical courses observed in monozygotic twins (34). Additional possibilities may also involve specific mutations affecting the Rett gene itself. Recently, investigators have identified a variety of mutations in the gene MECP2 encoding X-linked methyl-CpG-binding protein 2 (MeCP2), a trans-acting factor involved in regulating gene expression believed to cause RS in some cases (35). The nature of these mutations may contribute to patient heterogeneity by dictating whether there is either partial or complete loss of function of MeCP2.

The discovery of the RS gene will eventually make early detection and prenatal diagnosis possible. It will also help researchers begin to describe the pathogenesis involved in RS. MeCP2 interacts with a histone deacetylase complex to mediate transcriptional repression (35–37). Therefore, reduced function of MeCP2 would ostensibly result in an overproduction of certain genes. How this may ultimately affect NGF production is uncertain. However this investigation suggests that reduced NGF expression may be a possible consequence of MeCP2 gene mutation. Describing MECP2 mutation status will certainly be important in future RS research.

In another recent study the number of cortical ChAT-positive neurons were found to be significantly decreased in RS patients compared with controls (38). Since ChAT-positive cells are known to be influenced by the presence of NGF (39), it seems likely that reduced NGF levels would explain these findings. However this was not found to be the case in the same study in which ELISA was used in frozen sonicated tissue samples to determine NGF levels in 5 RS subjects and 7 controls. Although the mean of all 5 RS subjects was found to be reduced compared with the controls, the results did not achieve statistical significance. In an effort to explain these seemingly contradictory conclusions, 2 important possibilities exist. First, the study did not reveal individual values despite indicating a relatively large SD for frontal cortical RS values, which gives rise to the possibility that some RS subjects fall significantly below normal levels. Second, the sample size was significantly smaller than that used in the present study and may not have been large enough to influence the overall mean NGF value. Finally, since histology was not available, it was not possible to assess the level of astrogliosis, which we have shown to be critical in determining NGF expression in these patients.

The NGF receptor trkA OD values were also decreased in the RS group. Reduced trkA expression would most likely further reduce NGF activity in these patients. Chronic NGF exposure is believed to be necessary for the promotion and maintenance of neuronal trkA expression (40–42). Therefore, reduced trkA is likely a consequence of long-term developmental NGF insufficiency.

In summary, our results indicate that reduced NGF and its receptor trkA are involved in the pathogenesis of RS. Also, the evidence clearly suggests that injury signals as indicated by the presence of astrocytosis provide an alternative mechanism for the induction of NGF in these patients. Future investigations will focus on demonstrating reduced NGF levels in other brain areas, as well as in younger RS tissue closer to the onset of symptoms.

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