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Developmental profile of serum nerve growth factor levels in Rett complex

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Summary. - Rett syndrome (RS) is a progressive neurodevelopmental disorder predominant in females, characterised by mental deficiency, stereotyped hand-washing and apraxia. Notwithstanding the recent identification of the MECP2 gene likely involved in the pathogenesis of SR, the neurobiological bases of this syndrome are still largely unknown. Converging evidence shows that the brain levels of nerve growth factor (NGF), a neurotrophin regulating the development and functioning of central cholinergic neurons, are decreased in RS girls. In this study, the serum levels of NGF were measured in classic RS, in the preserved speech variant (PSV) and in normal controls. Overall analysis failed to evidence significant differences among the three groups. However, whereas NGF levels increased significantly with age in controls, the opposite profile was observed in classic RS, with a progressive age-dependent decrease of NGF. In PSV subjects NGF levels remained constant with age. These findings strengthen the hypothesis of NGF involvement in the pathogenesis of RS.

Key words: Rett syndrome, preserved speech variant (PSV), nerve growth factor, neurodevelopmental disorders.

Riassunto *(Profilo ontogenetico dei livelli sierici di* nerve growth factor *nella sindrome di Rett*). - La sindrome di Rett (SR) è un grave disordine neurologico a carattere progressivo che si manifesta nel sesso femminile entro il secondo anno di vita. Nonostante la recente identificazione del gene MECP2 verosimilmente coinvolto nella patogenesi della SR, le basi neurobiologiche della sindrome sono ancora sconosciute. Evidenze recenti indicano ridotti livelli di *nerve growth factor* (NGF), una neurotrofina che modula lo sviluppo e la funzione dei neuroni colinergici centrali, nel liquor e nella corteccia di soggetti Rett. In questo studio sono stati misurati i livelli di NGF nel siero di soggetti con SR (sia nella forma classica che in quella a linguaggio conservato) e in soggetti di controllo. I livelli complessivi di NGF non differivano significativamente tra i tre gruppi, ma contrariamente a quanto osservato nei controlli, i livelli sierici di NGF diminuivano significativamente con l'età nella SR classica, e si mantenevano costanti nella variante a linguaggio conservato. Questi risultati rafforzano l'ipotesi di un coinvolgimento del NGF nella patogenesi della SR.

Parole chiave: sindrome di Rett, variante a linguaggio conservato (PSV), *nerve growth factor*, disordini del neurosviluppo.

Introduction

Rett syndrome is a severe neurological disorder affecting predominantly females, characterized by the global deceleration of psychomotor development and the loss of acquired cognitive and motor skills, following an apparently normal or quasi-normal development during the first months of life [1, 2]. Rett syndrome is one of the most common causes of mental retardation in females, with an incidence of 1 in 10,000-15,000. The occurrence of stereotypic hand-washing activities and hand apraxia are among the more typical features of this condition which includes the failure to develop speech or the loss of all acquired speech during the so called regression phase. In fact the natural course of this disorder is depicted by a clinical staging system in four stages.

Rett syndrome is recognized today to have a broader variability in clinical phenotype than originally thought, at the point of suggesting the concept of a Rett Complex [3], including the classical RS as well as different variants which occasionally may group together in large family trees [4]. It is likely that the more frequent variant is represented by the preserved speech variant (PSV) where the course of the disorder is more benign and the occurrence of associated abnormalities (microcephalus, scoliosis, epilepsy, etc.) more rare than in classic RS [5-7]. Recently, mutations of the gene MECP2 (located at Xq28) have been identified in the majority of cases of sporadic classic Rett syndrome: this gene encodes the methyl-CpG-binding protein (MeCP2), which is critical for regulation of gene expression of an as yet unknown number and type of genes [8, 9]. De Bona *et al.* [10] have also shown that Rett PSV is allelic of classic Rett,

suggesting that phenotype variability is only partially dependent on the kind of MECP2 mutation and other mechanisms such as skewed X-inactivation and/or modifier gene effects could be responsible for the variable clinical output in Rett complex.

Neuropathological and neurochemical studies indicate that RS is a neurodevelopmental, rather than a neurodegenerative, disorder [11]. Notwithstanding the generalized brain atrophy no evidence of cell loss and degeneration was found in autoptic studies [12]: however, a consistent decrease in dendritic branching in different brain areas was found, especially pronounced at the frontal cortex level [13]. Moreover, there is evidence of a decreased number of basal forebrain cholinergic neurons in classic RS, paralleled by decreased ChAT activity in the hippocampus, thalamus and basal ganglia [11, 14]. These data suggest that there is a deficit in cholinergic function in RS, which might be associated with the severe cognitive impairments and extrapyramidal dysfunction [11]. It has been observed that levels of nerve growth factor (NGF), a neurotrophin exerting a crucial role in development and functional maintenance of forebrain cholinergic neurons [15, 16] are decreased in the cerebrospinal fluid of RS children and adolescents [17] as well as within the frontal cortex [18]. A potential role for NGF in the pathogenesis of neurodevelopmental disorders has also been supported by a recent study showing that NGF is severely decreased in the blood of young schizophrenic patients [19]. If, and to what extent, a change in the constitutive levels of brain NGF is reflected in the peripheral circulation is still a matter of debate. It has been clearly shown that NGF can cross the blood-brain barrier [20] whose permeability is higher in the early developmental phases. Moreover, since NGF acts as a link between different but functionally related systems, such as the nervous, endocrine and immune system [21], a correlation between central and peripheral NGF levels seems very likely. Given the ethical problems associated to the analysis of CSF in human subjects, it would be extremely important for both clinical and applied research to obtain information on neurotrophin levels in different pathological conditions by means of less invasive procedures.

In the present study the blood levels of NGF were measured in a cohort of girls with classic RS or with PSV, and compared to those of normal age-matched controls. Our aim was to verify: a) whether the levels of NGF were decreased in the peripheral circulation similarly to those previously reported in CSF for RS subjects [17], and b) whether such levels would differ in classic RS and in PSV.

Methods

Twentyseven girls affected by classic RS (age range = 3-22 yrs, mean age 8 years and 8 months) and nine girls affected by PSV (age range = 6-26 years, mean age 12 years and 4 months), attending the Department of Child Neuropsychiatry of the Siena's General Hospital, were the object of this study. Diagnostic criteria were those described by Trevathan and Moser [22] for classic RS and by Hagberg and Skjeldal [23] for the variants, including the description given for PSV by Zappella *et al*. [3]. The evaluation was conducted separately by two child neuropsychiatrists (M.Z. and J.H.) and results were concordant in every case. Sixteen healthy girls (age range = 3-22 years, mean age 9 years) not presenting neurological ilnesses or mental retardation were considered as controls. As PSV can be diagnosed only when language develops in these children (around 6 years), the age range of PSV group was larger than that of classic RS and control subjects.

Informed consent was obtained from the parents of patients after the nature and possible consequences of the study was explained. The study was approved by the Ethical Committee of the hospital.

Five milliliters peripheral blood samples were taken from each subject, collected in heparinized tubes and centrifuged at 2000 rpm for 20 min to separate serum from plasma, and stored at -20 °C until NGF determination. The levels of NGF were measured by a highly sensitive two-site immunoenzymatic assay which recognizes both human and murine NGF as previously described [24]. 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 non-specific signal. After an overnight incubation at room temperature and 2 hours incubation with a blocking buffer (0.05 M carbonate buffer pH 9.5+ 1% BSA), plates were washed with Tris-HCl pH 7.4 (50 mM, NaCl 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 hours at 37 °C and, after further washing, 100 ml of substrate solution (4 mg of chlorophenol red (Boehringer-Mannheim, Germany)/ml substrate buffer: 100mM HEPES, 150 mM NaCl, 2mM MgCl2, 0.1% sodium azide and 1% BSA) were added to each well. After an incubation of 2 hours at 37 °C the optical density was measured at 575 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 non-specific binding. Data are represented as pg/ml or pg/g wet weight and all assays were performed in triplicate. The exogenous NGF yield was calculated by subtracting the amount of this NGF from the endogenous variety. Under these conditions the recovery of NGF on our assay ranged from 80 to 90%. All values below the lower limit of detection of the assay were assigned a value of 1 pg/ml.

NGF serum levels were analysed by non parametric analysis of variance (Mann-Whitney U test), given the presence of cut-off values due to the detection limit of our assay (NGF undetectable if < 5 pg/ml). For this same reason, the correlation between age and NGF levels was analysed by the Spearman rank correlation coefficient (ρ_S) in each group.

Results

The serum levels of NGF did not differ significantly in the three groups (RS: median = 8 pg/ml, interquartile range = 1-16.6 pg/ml; PSV: median = 5.84 pg/ml, interquartile range = 1-19.65 pg/ml; controls: median = 7.09 pg/ml, interquartile range = 6.07-14.52 pg/ml). In nine patients with RS, in three patients with PSV and in two control girls the levels of NGF were below the limit of detection (less than 5 pg/ml). However, the correlational study applied to data to verify the potential relation between serum NGF levels and age, evidenced different ontogenetic trends in the three groups (Fig. 1). Specifically, whereas NGF values appeared to increase with age in normal controls (ρ_S = 0.4), in RS subjects these same values decreased with age (ρ_S = -0.3; comparison RS *vs* controls : $\chi^2 = 5.14$, $p < 0.05$). PSV girls displayed an intermediate profile between RS and control girls (ρ_S = -0.07).

In the Rett complex, both in the classic RS and in the variants, including PSV, a sequence of subsequent stages is the rule, accompanied by progressive deterioration which becomes maximal for stage 4: in the present series, however, there was not a clear correlation with the stages or with the ability to walk (8 RS girls and one PSV were not ambulant).

Discussion

Converging lines of evidence indicated that neurotrophic factors are important regulators of brain development. In particular NGF, the best known and characterised among them, exerts a pivotal role in the maturation and neurochemical differentiation of basal forebrain cholinergic neurons [16]. As indicated by a variety of animal studies, lack of NGF support at the appropriate developmental phase results in derailment of cholinergic function and in behavioural abnormalities,

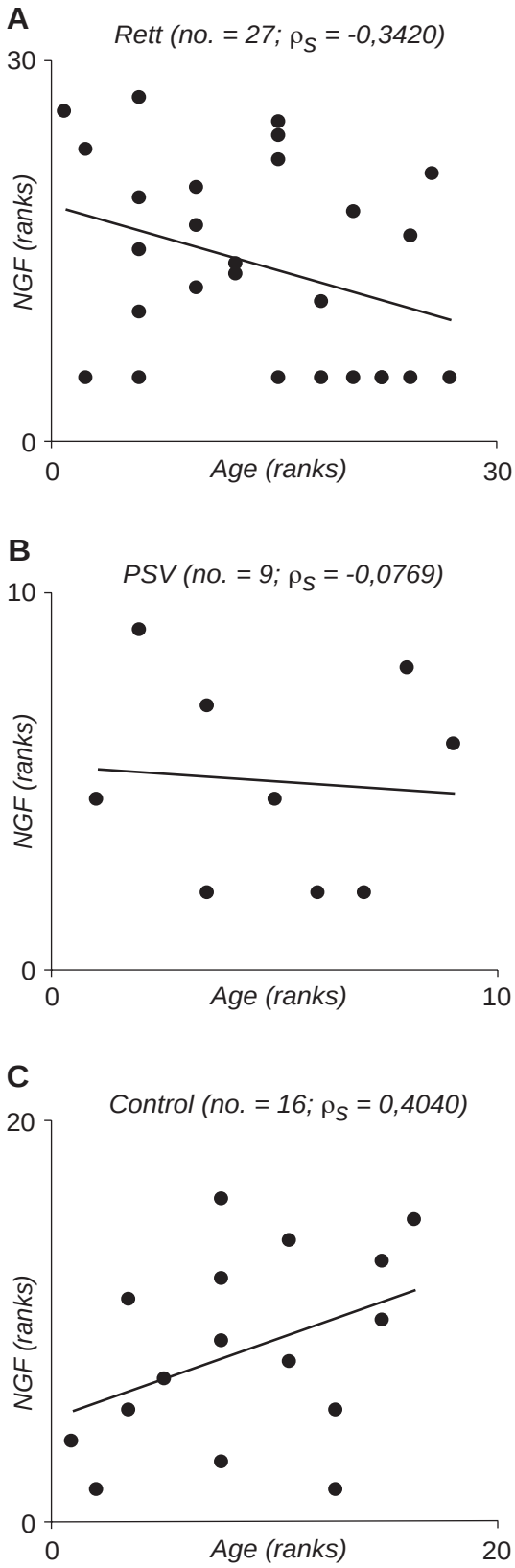

Fig. 1. - Scatterplots of ranked data concerning age and serum NGF values for girls with classic Rett syndrome (A), with preserved speech variant (B), and for control girls (C). The line trends for each group are also plotted. ρ_S = Spearman correlation coefficient.

including impaired learning and memory capacities [25-27]. It is thus likely that the altered cholinergic function, as well as the reduction in number and size of forebrain cholinergic neurons observed in RS, might be associated to lack of appropriate trophic support by NGF and/or other neurotrophins during early brain development [28]. The recent report by Lipani *et al.* [18], indicating reduced expression of NGF protein and of its receptor within the frontal cortex of RS subjects, strongly supports the involvement of this neurotrophin in the pathogenesis of RS.

In the present study, the decrease of serum levels of NGF in the Rett Complex appears to be related to subjects' age, being more evident in classic RS than in PSV. In classic RS girls reduced levels of NGF were matched with CSF normal levels of NGF in two recent studies [17, 29] suggesting that NFG may have low levels within the central nervous system. However, Vanhala *et al.* [29] did not find decreased NGF in the serum of RS subjects, nor they evidenced any significant correlation with age. Our data, showing that the overall NGF levels in both RS and PSV subjects were not significantly different from those of controls, partly confirm Vanhala's findings. Yet, we found a peculiar developmental trend in RS girls, with a tendency to a marked reduction of circulating NGF with increasing age. NGF values in PSV girls appeared to remain constant with age, and this might be consistent with the more benign course of this disorder compared to RS. The apparent discrepancy of our results from those of Vanhala *et al.* [29] might be due to the different sample size (12 RS subjects in Vanhala's study *vs* 27 subjects in our study). Moreover, our control group was represented by 16 healthy female subjects, while Vanhala *et al.* included in the control group both male and female subjects with various pathological conditions. It must be noticed that in Lappalainen *et al.* report [17] five out of six RS girls older than 11 years had no noticeable levels of NGF in the CSF, in contrast with higher values for younger girls. Thus it appears that, when considering the developmental profile, the serum levels may to some extent reflect CSF levels and are possibly related to CNS. The discovery of the RS gene will eventually help researchers to understand the pathogenetic mechanisms involved in RS. MECP2 interacts with a histone deacetylase complex to mediate transcriptional repression [8], and thus reduced function of MECP2 would result in an overproduction of certain genes. Whether and to what extent this may affect NGF synthesis and expression it is still unclear.

On the whole, the present findings are in line with number of studies that have reported altered NGF levels in the CSF or serum of various child and adolescent clinical conditions, including schizophrenia [19], West syndrome [30], and progressive cortical atrophy [31]. Moreover, altered serum levels of NGF have been found in a genetically-based mental retardation syndrome: in Williams syndrome, for example, they persist at higher levels through childhood and adolescence in comparison to normal age-matched controls [32]. It therefore appears that the alteration of this neurotrophin plays a role in the pathogenesis of neurodevelopmental disorders. The relationship between peripheral and brain NGF levels under normal and pathological conditions needs to be further explored, also in the light of the obvious advantage to monitor NGF and other neurotrophin levels in the developing organism by means of non-invasive procedures.

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REFERENCES

1. Hagberg B, Aicardi J, Dias K, Ramos O. A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls. Rett syndrome: a report of 35 cases. *Ann Neurol* 1983;24:383-8.

2. Rett A. Ueber ein eigenartiges hirnatrophisches Syndrom bei Hyperammonaemie im Kindesalter. *Wien Med Wochenschr* 1966;116:723-38.

3. Zappella M. The preserved speech variant, a subgroup of the Rett complex: a clinical report of 30 cases. *J Aut Devel Dis* 1998; 28:519-26.

4. Pini G, Milan M, Zappella M. Rett syndrome in Northern Tuscany (Italy): family tree studies. *Clin Genet* 1996;50:486-90.

5. Zappella M. The Rett girls with preserved speech. *Brain Dev* 1992;14:98-101.

6. Zappella M. Rett syndrome-like hand-washing, developmental arrest and autistic symptoms in two Italian girls. *Eur Child Adolesc Psych* 1994;3:52-6.

7. Zappella M. The preserved speech variant of the Rett complex: a report of 8 cases. *Eur Child Adolesc Psych* 1997;6:23-5.

8. Amir RE, Van der Veyver IB, Wan M, Tran CQ, Franke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet* 1999;23:185-8.

9. Percy AK. Genetics of Rett syndrome: properties of the newly discovered gene and pathobiology of the disorder. *Curr Op Ped* 2000;12:589-95.

10. De Bona C, Zappella M, Hajek G, Meloni I, Vitelli F, Bruttini M, Cusano R, Loffredo P, Longo I, Renieri A. Preserved speech variant is allelic of classic Rett syndrome. *Eur J Hum Genet* 2000;8:325-30.

11. Wenk GL. Rett syndrome: neurobiological changes underlying specific symptoms. *Progr Neurobiol* 1997;51:383-91.

12. Bauman ML, Kemper TL, Arin MD. Microscopic observations of the brain in Rett syndrome. *Neuropediatrics* 1995;26:105-8.

13. Belichenko PV, Dahlstrom A. Confocal laser scanning microscopy and 3D reconstructions of neuronal structures in human brain cortex. *Neuroimage* 1995;2:201-7.

14. Naidu S. Rett syndrome: a disorder affecting early brain growth. *Ann Neurol* 1997;42:3-10.

15. Levi-Montalcini R. The nerve growth factor 35 years later. *Science* 1988;237:1154-62.

16. Mobley WC, Rutkowsky JL, Tennekoon GI, Gemski J, Buchanan KM, Johnston MV. Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Mol Brain Res* 1986;1:53-62.

17. Lappalainen R, Lindholm D, Riikonen R. Low levels of nerve growth factor in cerebrospinal fluid of children with Rett syndrome. *J Child Neurol* 1996;11:296-300.

18. Lipani JD, Bhattacharjee MB, Corey DM, Lee DA. Reduced nerve growth factor in Rett syndrome postmortem brain tissue. *J Neuropathol Exp Neurol* 2000;59:889-95.

19. Bersani G, Aloe L, Iannitelli A, Maselli P, Venturi P, Garavini F, Angelucci F, Bracci Laudiero L, Pancheri P. Low nerve growth factor (NGF) plasma levels in schizophrenic patients: a pilot study. *Schiz Res* 1996;18:159-60.

20. Poduslo JF, Curran GL, Berg CT. Macromolecular permeability across the blood-nerve and blood-brain barriers. *Proc Natl Acad Sci USA* 1994;91:5705-9.

21. Levi-Montalcini R, Aloe L, Alleva E. A role for nerve growth factor in nervous, endocrine and immune systems. *Progr Neuroend Immunol* 1990;3:1-10.

22. Trevathan E, Moser HW. Diagnostic criteria for Rett syndrome. *Ann Neurol* 1988;23:425-8.

23. Hagberg B, Skjeldal OH. Rett variants: a suggested model for inclusion criteria. *Pediatr Neurol* 1994;11:5-11.

24. Bracci-Laudiero L, Aloe L, Levi-Montalcini R, Buttinelli C, Schilter D, Gillessen S *et al.* Multiple sclerosis patients express increased levels of nerve growth factor in cerebrospinal fluid. *Neurosci Lett* 1992;147:9-12.

25. Alleva E, Aloe L, Calamandrei G. Nerve growth factor influences neurobehavioural development of newborn mice. *Neurotoxicol Teratol* 1987;9:271-5.

26. Calamandrei G, Alleva E. Neuronal growth factors, neurotrophins and memory deficiency. *Behav Brain Res* 1995;66:129-32.

27. Calamandrei G, Ricceri L, Valanzano A. Systemic administration of anti-NGF antibodies to neonatal mice impairs 24-h retention of an inhibitory avoidance task while increasing ChAT immunoreactivity in the medial septum. *Behav Brain Res* 1996; 78:81-91.

28. Wenk GL, O'Leary M, Nemeroff CB, Bissette G, Moser H, Naidu S. Neurochemical alterations in Rett syndrome. *Dev Brain Res* 1993;74:67-72.

29. Vanhala R., Korhonen L, Mikelsaar M, Lindholm D, Riikonen R. Neurotrophic factors in cerebrospinal fluid and serum of patients with Rett syndrome. *J Child Neurol* 1998;13:429-33.

30. Riikonen RS, Söderström S, Vanhala R, Ebendal T, Lindholm DB. West syndrome: cerebrospinal fluid nerve growth factor and effect of ACTH. *Pediatr Neurol* 1997;17:224-9.

31. Suzaki I, Hara T, Tanaka C, Dejima S, Takeshita K. Elevated nerve growth factor levels in cerebrospinal fluid associated with progressive cortical atrophy. *Neuropediatrics* 1997;28:268-71.

32. Calamandrei G, Alleva E, Cirulli F, Queyras A, Volterra V, Capirci O, Vicari S, Giannotti A, Turrini P, Aloe L. Serum NGF levels in young children, children and adolescents with Williams and Down syndrome. *Dev Med Child Neurol* 2000;42:746-50.