Serum NGF levels in children and adolescents with either Williams syndrome or Down syndrome

Gemma Calamandrei PhD;
Enrico Alleva PhD;
Francesca Cirulli PhD;
Armelle Queyras, Laboratorio di Fisiopatologia di Organo e di Sistema, Istituto Superiore di Sanità;
Virginia Volterra PhD;
Olga Capirci PhD, Institute of Psychology, Consiglio Nazionale delle Ricerche, Rome;
Stefano Vicari MD, IRCCS Children's Hospital Bambino Gesù, Santa Marinella;
Aldo Giannotti MD, IRCCS Children's Hospital Bambino Gesù, Rome;
Paolo Turrini;
Luigi Aloe* PhD, Institute of Neurobiology, Consiglio Nazionale delle Ricerche, Viale C. Marx 15, I-00137 Rome, Italy.

**Correspondence to last author at* Institute of Neurobiology, Consiglio Nazionale delle Ricerche, Viale C. Marx 15, I-00137, Rome, Italy.
E-mail: aloe@in.rm.cnr.it

The neurotrophin nerve growth factor (NGF) is a major regulator of peripheral and central nervous system development. Serum NGF was measured in normally developing control children (n=26) and in individuals affected by congenital syndromes associated with learning disability: either Williams syndrome (WS; n=12) or Down syndrome (DS; n=21). Participants were assessed at three distinct developmental stages: early childhood (2 to 6 years), childhood (8 to 12 years), and adolescence (14 to 20 years). A sample was taken only once from each individual. Serum NGF levels were markedly higher in participants with WS, than DS and control participants. In addition, different developmental profiles emerged in the three groups: while in normally developing individuals NGF levels were higher in early childhood than later on, children with WS showed constantly elevated NGF levels. When compared to control participants, those with DS showed lower NGF levels only during early childhood. Neuropsychological assessment confirmed previously reported differences among the three groups in the development of linguistic/cognitive abilities. Some features of individuals with WS, such as hyperacusis and hypertension, could be related to high-circulating NGF levels.

Williams syndrome (WS) is a rare genetic condition (1: 20 000) characterized by a distinctive clinical pattern which includes supravalvular aortic stenosis, growth deficiency, learning disability, and so-called elfin/pixie facial features (Williams et al. 1961). In all recently reported cases of WS, genetic studies have identified a deletion on the long arm of chromosome 7, including the elastin and the LIMK1 genes (Ewart et al. 1993, Frangiskakis et al. 1996). WS has recently received attention for the apparent dissociation between cognitive and linguistic abilities described by some authors (Bellugi et al. 1990, Wang and Bellugi 1994). Down syndrome (DS) is the most common form of learning disability with a genetic cause, namely trisomy of chromosome 21. DS has a prevalence of 1 in 800 births and it is clinically manifested by somatic anomalies, heart diseases, learning disability, and precocious dementia (Scott et al. 1983). Individuals with both WS and DS have cognitive impairments, but distinct cognitive profiles of the two groups emerge from the comparison of their linguistic and visuospatial functioning (Wang and Bellugi 1993).

The hypothesis underlying this study is that the altered profile of neurobehavioural development found in those with WS and DS may be associated with altered levels of nerve growth factor (NGF) expression in various bodily compartments during critical developmental stages. NGF is the first and best characterized neurotrophin (Levi-Montalcini 1987). In addition to having a trophic, tropic, and differentiating effect on peripheral neurones, NGF also regulates development and survival of forebrain cholinergic neurones (Mobley et al. 1986, Levi-Montalcini 1987). Animal studies have shown that alterations in endogenous NGF levels during the early postnatal stage influences the development of learning and memory capacities in rodents (Calamandrei et al. 1996). As for human studies, low levels of NGF have been detected in the CSF of children with Rett and West syndromes: two neurological disorders associated with mental impairment (Lappalainen et al. 1996, Riikonen et al. 1997, Riikonen and Vanhala 1999). It has been suggested that altered production of specific neurotrophins during critical developmental phases may be involved in the pathogenesis of some psychiatric disorders as well as of syndromes associated with learning disability (Bersani et al. 1996).

In this interdisciplinary study, we measured circulating NGF levels in the serum of individuals with WS, DS, and normally developing control participants at three different postnatal ages: early childhood, childhood, and adolescence. Neuropsychological profiles of the three groups were also compared, focusing on the development of linguistic and visuospatial abilities.

Method

Participants for this study were 21 individuals with DS and 12 individuals with WS, sex-matched, belonging to three age groups: (1) 2 to 6 years old (young children); (2) 8 to 12-years old (children); and (3) 14 to 20 years old (adolescents, see Table I). Each participant was tested only once. All participants were involved in a larger study on medical and neuropsychological features of WS conducted at the Bambino Gesù children's hospital in Santa Marinella and in Rome, Italy. Participants with WS and DS were diagnosed by a paediatric geneticist on the basis of major stigmata; the

diagnosis was confirmed by molecular analysis. Control participants ($n=26$) were matched with individuals with WS and DS either for chronological or mental age. For physiological data (NGF levels) control participants were matched for chronological age, while for the neuropsychological analysis, they were matched for mental age. In the case of young children, mental and chronological age coincided. Control participants were healthy individuals presenting with neither neurological illness nor learning disability. Participants were included in the study only after routine blood testing had excluded infective and/or inflammatory states. Informed consent was obtained from the participants' parents after the nature and of the study were explained.

Peripheral blood samples of 5 mL were taken from each participant between 08.00 h and 10.00 h, 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. NGF levels were measured by a highly sensitive two-site immunoenzymatic assay which recognizes both human and murine NGF as previously described (Weskamp and Otten 1987). The sensitivity of this assay is 5 pg/mL, i.e. the minimum concentration of NGF detectable. Data are represented as pg/mL or pg/gr wet weight; 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 arbitrarily assigned a value of 1 pg/mL.

Linguistic and visuospatial abilities were tested in each participant on 2 or 3 different days. Neuropsychological assessment was performed by using an experimental battery composed of various tests designed to investigate linguistic and visuospatial abilities in the different age groups, which have been employed in previous studies to describe cognitive profiles of participants with DS, WS, or with learning disability of different aetiology (see Vicari et al. 1992 and Volterra et al. 1996, for a detailed explanation of the linguistic and visuospatial tests used in the present study).

The communicative and linguistic development of the younger children included in this study (group 1) was measured by administering the Italian MacArthur questionnaire to parents. As for participants belonging to groups 2 and 3, their linguistic abilities were assayed using the following tests: (1) Brizzolara Naming Test, to explore participants' ability to produce verbal labels for common nouns (e.g. animals, flowers); (2) Word Fluency (phonemic) Test: three letters of the alphabet (f, a, s) were read sequentially to the participant who was asked to say as many words as possible that begin with that letter, excluding proper nouns, numbers, and the same word with a different suffix; and (3) Word Fluency (semantic) Test, to explore semantic word retrieval ability using four superordinate category names: animals, toys, food, and clothes. Given the name of a category, the participant was asked to generate as many items as they could think of within that category. In addition, the widely-used Peabody Picture Vocabulary Test (PPVT), was selected to provide a reliable measure of lexical comprehension and the Visual Motor Integration Test (VMI) was administered to test participants' visuoconstructive abilities.

STATISTICAL ANALYSIS

Data on NGF levels were analyzed using the non-parametric Kruskal–Wallis analysis of variance for independent groups. To assess the significance of main effects of chronological age, condition (WS, DS, control) and their interactions, the χ^2 test was applied. Multiple comparisons were performed by Mann–Whitney tests with Bonferroni correction. Data of comparisons between groups are presented as median difference and 95% confidence interval (95% CI) of the median. An experimentwise type I error probability of 10% was considered statistically significant for multiple comparisons. Such a lenient criterion (experimentwise $p=0.1$) is appropriate to avoid making too many type II errors with a large number of comparisons involving a relatively small number of participants. The results of the neuropsychological tests were analyzed by means of non-parametric tests (Wilcoxon matched-pair test).

Results

Overall, NGF levels differed significantly in the different groups ($\chi^2=24.15$, $df=2$, $p=0.00001$; Fig. 1). Post-hoc comparisons showed that NGF levels in participants with WS were significantly elevated compared to both individuals with DS and normally developing control participants ($p<0.05$). Neither a main effect of age ($\chi^2=2.92$, $df=2$, $p=0.23$), nor a significant interaction between age and group were found (age × group $\chi^2=9.00$, $df=4$, $p=0.06$). Nonetheless, post hoc comparisons revealed that in the first age group (2 to 6 years old), children with DS showed significantly lower NGF levels compared with both those with WS (30.5 pg/mL, 95% CI 16.0 to 82.0, $p<0.1$) and control individuals (17.0 pg/mL, 95% CI 9.4 to 27.4, $p<0.025$). Although individuals with WS tended to have higher NGF

Table I: Sex and age of participants involved in the study

Williams syndrome		*Down syndrome*		*Control group*	
Sex	*Age (y, mo)*	*Sex*	*Age (y, mo)*	*Sex*	*Age (y, mo)*
M	2, 5	M	2, 6	M	2, 0
F	2. 6	F	2, 8	M	2, 3
M	2, 6	M	2, 8	F	2, 7
F	4, 9	F	3, 8	M	4, 3
F	8, 9	M	4, 10	F	4, 7
F	9, 2	F	5, 1	F	4, 11
M	10, 1	F	5, 3	F	6, 0
M	12, 4	M	8, 8	M	6, 1
M	17, 6	F	9, 0	F	6, 2
F	18, 3	F	9, 2	F	6, 2
F	18, 10	F	9, 6	M	6, 3
M	19, 8	M	9, 9	M	6, 10
		M	10, 5	F	7, 1
		M	11, 11	F	7, 11
		M	15, 6	F	8, 2
		F	15, 10	F	8, 3
		M	17, 1	F	9, 2
		F	17, 8	M	9, 5
		F	17, 10	F	10, 6
		F	19, 9	M	11, 10
		M	19, 9	F	13, 9
				M	14, 5
				F	17, 6
				M	17, 10
				F	18, 2
				M	18, 5

levels than control participants, this difference was not significant. In the second age group (8 to 12 years old), the levels of NGF were significantly elevated in WS compared with both DS (21.7 pg/mL, 95% CI 10.6 to 46.5; $p<0.1$) and control participants (23.5 pg/mL, 95% CI 11.6 to 50.5; $p<0.1$), while DS and control individuals did not differ from each other. In the third age group (14 to 20 years old), the same pattern of NGF expression was maintained (with NGF levels higher in WS than in DS and the control group), although differences between groups failed to reach significance. Finally, NGF levels of the control group matched for mental age did not differ from those of the control group matched for chronological age.

Results of the neuropsychological analysis also showed different developmental patterns of the three groups, in particular when considering the second and third age groups (Table II). In the younger age group (2 to 6 years) the mean number of words produced by participants with DS and WS was very similar (135 and 133.25 respectively) although significantly lower ($p<0.05$) compared with their chronologically-matched control participants (436.6). This finding is consistent with previous reports describing an initial delay in lexical development both for children with DS and WS. As for the second and third age group, no differences emerged when comparing the scores of control individuals matched for mental age with participants with WS and DS in the Lexical Comprehension test (PPVT test, see Table II). WS participants obtained scores comparable to those of their normally-developing control participants matched for mental age in both Naming and Phonemic Fluency tests (see Table II), while children and adolescents with DS had worse performances than control and participants with WS in these same tests ($p<0.5$). When requested to produce names belonging to particular semantic categories, participants with DS and WS did not differ from normally-developing mental-age matched control participants (see Table II). Similarly, performances of participants with DS and WS were comparable to those of the control group in the Visual Motor Integration Task.

Table II: Comprehension, word production, phonemic and semantic test results for participants in age groups 2 and 3.

Test	*Mean score (SD)*	*Range*
Peabody Picture Vocabulary Test		
WS ($n=8$)	73 (22.5)	39–96
DS ($n=8$)	57.5 (20.1)	30–84
MA ($n=5$)	80.8 (15.5)	59–94
Naming Test		
WS ($n=8$)	67.9 (14.3)	51–93
DS ($n=5$)	42 (10.9)	22–54
MA ($n=5$)	70.8 (6.9)	68–82
Phonemic Fluency Test		
WS ($n=7$)	15.4 (7)	5–28
DS ($n=7$)	4.9 (3.1)	2–10
MA ($n=5$)	12.8 (6.5)	4–21
Semantic Fluency Test		
WS ($n=7$)	34.9 (3.8)	29–39
DS ($n=7$)	25.7 8.2)	14–37
MA ($n=5$)	33.4 (9)	25–44

WS, Williams syndrome; DS, Down syndrome; MA, control participants matched for mental age.

Discussion

The present findings reveal that serum NGF levels of participants with WS were significantly higher than those measured in DS and control participants. In addition, when data were analysed, taking into account participants' age, different developmental profiles emerged in the three groups.

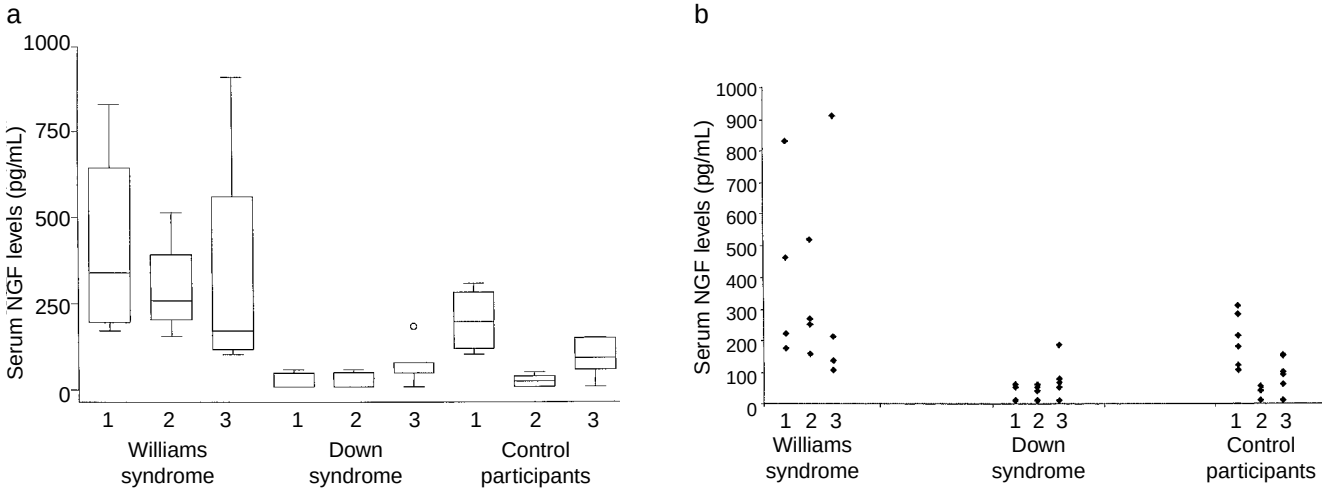

Figure 1: *Serum NGF levels (pg/mL) of individuals with WS, DS and controls (matched for chronological age, CA; WS, n=4; DS, n=7; CA, n=6) in three age groups. Age group 1, 2 to 6 y; group 2, 8 to12 y; group 3, 14 to 20 y. In age group 1 NGF levels of participants with DS differed significantly from both WS and control groups. In age group 2, participants with WS showed significantly higher NGF levels than both those with DS and control participants. (a) Box and whisker plot. Line in middle of box represents median. Box covers interquartile range (IQ); whiskers extend to upper and lower adjacent values. Upper value is defined as largest data point less than or equal to* $x_{[75]} + 1.5 \times IQ$. *Lower value is defined as smallest data point greater than or equal to* $x_{[25]} - 1.5 \times IQ$. *Outliers, ○, (points more extreme than the adjacent values). (b) Scatter plot of the same data.*

Specifically, while NGF levels in control participants were high in early childhood and tended to decrease with age, in individuals with WS the levels of this neurotrophin remained high from early childhood to adolescence. In contrast, serum NGF concentrations in young children with DS were lower than those of control participants, but reached levels similar to those of age-matched control individuals in later childhood and adolescence. To explain the physiological significance of altered circulating NGF levels requires careful consideration of the role of NGF in the development and specific pathological phenotype of WS. Indeed, during the pre- and postnatal period, normal development of peripheral sympathetic and sensory neurones depends on the availability of NGF produced by their target organs, which controls cell growth, neurite production and elongation, and selective cell death or survival. However, maintenance of abnormally high levels of NGF throughout development might induce sympathetic hypertrophy, hyperinnervation of target organs, and general nerve dysfunction as well as hormonal and immunological alterations (Aloe et al. 1981). In participants with WS, the overall trend is that of a permanently elevated NGF level in the bloodstream. We suggest that this increase is indicative of a more general increase in NGF expression, that might have affected developmental processes in different parts of the body.

If this is the case, some of the characteristic clinical features of WS might be connected to the altered NGF levels. A signature symptom of WS is hyperacusis, a heightened sensitivity to sound (Dilts et al. 1990). Such a peculiar hypersensitivity to sounds might depend upon a peripheral 'hyperinnervation' of the internal ear due to an excess of NGF production by the target organ. It has been previously shown that neurotrophins and their receptors are expressed by auditory hair cells in rats' inner ears during target innervation, and that a decrease of NGF causes an inhibition of neuronogenesis in the cochleovestibular ganglion (Staecker et al. 1996). Thus, changes in the expression of NGF during development and at the adult stage could affect the innervation of the inner ear, possibly affecting auditory performance. Although there is less evidence for heightened sensitivity to stimulation on other modalities of patients with WS, anecdotal evidence suggests that individuals with WS are exceptionally sensitive to touch on the extremities (i.e. the fingers and toes), a phenomenon which could be related to reports of motor difficulties in the first year of life. Changes in the peripheral sensory innervation as a result of changes in NGF availability in the innervation targets may explain some of these features. In developing rodents, NGF is critical for the normal development and function of nociceptive sensory neurones (Lewin et al. 1994), whereas systemic administration of NGF produces dose-dependent hyperalgesia in humans (Anand 1995). Moreover, a potential link between the hypertension observed in individuals with WS and increased NGF levels may also be suggested, as changes in circulating and renal NGF levels seem to be implicated in the pathogenesis of hypertension (Lee et al. 1987).

High levels of NGF found in the blood of control participants in early childhood may be physiologically significant as this is a phase of intense synaptogenesis and of maturation of sensory integration. Overall, serum NGF levels of participants with DS did not differ from those of normally developing control participants. However, participants with DS appear to display a distinctive developmental profile, with NGF levels being lower than in control participants in early infancy. Changes in brain synaptic development and morphology in the pre- and postnatal phases have been reported in DS, with precocious cessation in the development of dendritic spines (Scott et al. 1983), while in the periphery, alterations of electric membrane properties of dorsal root ganglia neurones have been recorded (Scott et al. 1983). The low NGF levels present in the bloodstream of young children with DS might be related to previously reported alterations in the peripheral nervous system.

Results of the neuropsychological analysis confirmed previously reported differences among the three groups in the development of linguistic/cognitive abilities (Vicari et al. 1992, Wang and Bellugi 1993, Bishop 1999). When considering the first age group, a relevant difference between participants with WS and DS was that participants with DS displayed a developmental pattern similar to normally developing participants: they used communicative gestures and understood many words before producing their first words. In contrast participants with WS did not use gestures pre-linguistically and they produced more words than they comprehended. As for the two older age groups, it appears that participants with WS do not show a homogeneous developmental delay, but rather an asynchronic and atypical pattern of development of different abilities (Fabbretti et al. 1997, Jarrold et al. 1998). In this study participants with DS, similarly to control participants, performed better in the Semantic Fluency test than in the Phonemic Fluency test (Volterra et al. 1996). Children and adolescents with WS exhibited the opposite pattern: their performance in the Phonemic fluency test was even better than the performance exhibited by normally developing control participants matched for mental age.

On the whole, the increased circulating NGF levels found in participants with WS is indicative of a general alteration of developmental processes which occur outside the brain. However, as WS is a disorder also characterized by brain deficits, a key question raised by our observations is the functional relevance of changes in the levels of NGF in the peripheral circulation and in the CNS. In the absence of CSF measurements, it is hard to say whether or not central NGF levels are correspondingly altered. If, and to what extent, NGF produced by peripheral cells is transported into the CNS, and how much of the NGF produced in the CNS passes into the peripheral blood circulation is still a matter of debate, although recent studies have shown that NGF can cross the blood–brain barrier (Poduslo et al. 1994). Furthermore, as NGF acts as a link between different, although functionally related, systems, such as the nervous, endocrine, and immune system (Levi-Montalcini et al. 1990) we cannot exclude that changes in levels of this neurotrophin in the periphery may reflect an analogous alteration in the CNS. These data are to be considered as preliminary findings, and further studies involving many more participants are needed to verify the potential involvement of NGF and other neurotrophins in the pathophysiological features of Williams yndrome and Down syndromes.

Conclusion

The present study represents the first evidence of an alteration in peripheral NGF levels in a genetically-based syndrome with learning disability. Together with data indicating

low NGF levels in the CSF of participants with Rett syndrome, these findings suggest the involvement of this neurotrophin in the pathophysiology of neurodevelopmental disorders and indicate the need for further investigation.

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