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Enlargement of GAD-immunopositive terminals in the lateral vestibular nucleus (LVN) of Weaver mutant mice

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Summary. Reorganization of cerebellar circuitry due to specific cell loss in Weaver mutants causes physiological and morphological alterations in the terminal domains of the GABAergic cerebellar corticovestibular projections. In this study sizes of anti-GAD immunopositive terminals in the dorsal part of the lateral vestibular nucleus (dLVN) of normal mice and Weaver mutants were quantified morphometrically. In anti-GAD-immunoreacted material terminal sizes in the dLVN of Weaver exceed significantly those of coprocessed wildtypes. This suggests that the cerebellar disturbances in Weaver predispose the normal synaptic remodelling observed in wildtypes towards the formation of enlarged terminals.

Key words: Weaver mutant, Cerebellum, Lateral vestibular nucleus, Anti-GAD, Terminal size

Introduction

Synaptic remodelling is described to occur under such conditions as development, adaptation, degeneration or aging (Sotelo and Palay, 1971; Johnson and Miquel, 1974; Cotman, 1985). In healthy adult rodents remodelling is reported to be performed throughout their entire life span (Sotelo and Palay, 1971; Bäurle et al., 1992) and is considered to reflect, to a certain degree, the continued potency of the mature brain to react and adapt to altered extrinsic and intrinsic influences (Cotman, 1985). Moreover, remodelling in terms of collateral or expansional sprouting of neighbouring homo- or heterotypical presynaptic elements as a consequence of brain lesions can lead to the reoccupation of vacated postsynaptic space, as observed under various experimental conditions (Cotman, 1985; Bäurle et al., 1992).

In the Weaver mutant (Lane, 1965), which can be considered as an animal model of cerebellar ataxia, the almost complete loss of premigratory cerebellar granule cells (degeneration from postnatal day 1 (P1) until P14 (Smeyne and Goldowitz, 1989)) and the partial loss of Purkinje cells (PC) (50% in the vermis (Blatt and Eisenman, 1985)) lead not only to disturbances in cerebellar histology (Sotelo, 1982) but also to morphological and functional alterations in the vestibular nuclei (Grüsser-Cornehls, 1988), parts of which receive massive GABAergic Purkinje cell input (De Camilli et al., 1984).

To investigate the influence of these conditions on the size of GABAergic terminals in the dLVN, we combined the high amplification properties of the Avidin-biotin-method (ABC) (Hsu and Ree, 1980) and the poor leakage of the GAD-enzyme (glutamic-acid decarboxylase) to quantify GAD-immunopositive terminals (Mc Laughlin et al., 1974) according to size in the dLVN of Weaver mutants and normal controls. Preliminary results of this investigation have been published in abstract form (Krug and Grüsser-Cornehls, 1991; Krug et al., 1991).

Materials and methods

A total of 12 mice (6 Weaver mutants (wv/wv) and 6 B6CBA-wildtypes (+/+)) of both sexes, ranging in age from 5-6 months, were used for GAD-immunocytochemistry. All animals were raised in our colony on a natural dark/light rhythm and high quality food and water ad libitum; the parents originated from the Jackson Laboratories (Bar Harbor, Maine, USA).

The animals were sacrificed with a lethal dose of chloral-hydrate (1.75 g/kg body weight; Merck, No. 2425). Transcardial perfusion was initiated with warm normal saline (0.9%) buffered in 0.067M phosphate buffer at pH 6.5 for the incubation with the GAD-antiserum. The pH values and the concentration of the fixative were determined by preliminary tests in order to obtain optimal immunostaining of axon terminals (see also Mugnaini and Oertel, 1985). Fixation was performed with 1% paraformaldehyde (Merck No. 4005) and 1% glutaraldehyde (Merck, No. 4239) in 0.12M phosphate buffer at the same pH values as above for

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30 min.

The GAD-antiserum, a gift from Dr. Oertel, was raised in sheep; its properties are fully described (Oertel et al., 1983). The GAD-antibody was used at a dilution of 1:1000. After 2 hours in the same fixative, the brains were cut in the coronal plane at 30 µm on a vibratome (TPI Series 1000). Immunocytochemistry was performed according to a standard protocol of the ABC-method (Hsu and Ree, 1980).

Camera lucida drawings were made of immunopositive terminals found in association with «giant» cells in the dLVN using oil-immersion objectives (plan-apo x63, n.a.=1.4 and plan x100 n.a.=1.3, phase contrast and bright field). The maximum cross-sectional area of the terminals was measured with an image analyzing system (Kontron, Zeiss) interfaced with a micro-computer. Terminal sizes were grouped into categories using bin widths of 0.2 μm^2.

To test for the significance of differences in distribution three different statistical procedures were chosen, the results of which are given in Table 1.

Results

Fig. 1 shows the GAD-immunopositive terminals innervating immunonegative cell bodies of giant cells in

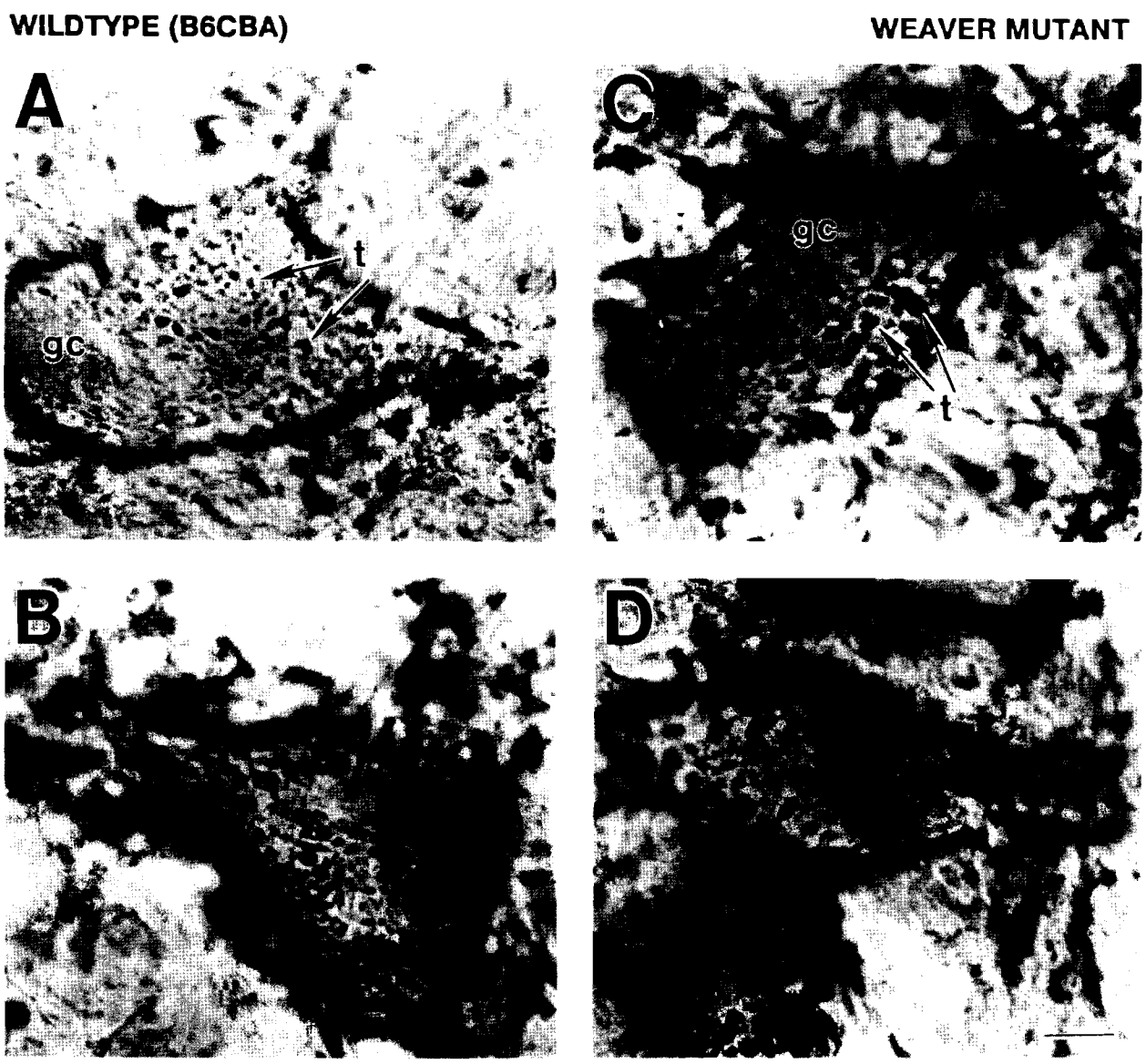

Fig. 1. Anti-GAD immunopositive terminals in the dLVN of coprocessed wildtypes **(A, B)** and Weaver mutants **(C, D)**. The somatic circumference of giant cells (gc) is contured by the dense innervation of GAD-immunopositive terminals. Terminals (t) synapsing from underneath the immunonegative cells are clearly visible in their full dimensions. Note the terminal size differences between wildtype and Weaver mutant. x 1,600. Bar= 6.25 µm.

the dLVN of normal mice (A, B) and Weaver mutants (C, D). Although photographs do not allow separate focusing of individual terminals, it is possible even without size quantification to recognize the enlarged synaptic boutons in the Weaver mutant.

The results of the morphometric analysis of the maximum cross-sectional areas of immunopositive terminals are given in Figs. 2 and 3. More than 9000 terminals were evaluated. Fig. 2 shows a comparison of individual animals and Fig. 3 the mean value curves of all animals investigated. The size distributions of GAD-immunoreacted terminals in mutants show significantly larger terminals compared to wildtypes. No differences were detected between wildtypes themselves or mutants themselves incubated with anti-GAD (not shown).

To obtain substantial evidence about the significance of the differences a set of statistical tests was chosen. The first test used was the t-test, which is based on the assumption of a normal distribution. However, as is visible from Figs. 2 and 3, the terminal sizes are not exactly normally distributed. To take this into account, the non-parametric Wilcoxon-test was performed in addition. As demonstrated in Table 1, both tests reveal significant differences with comparable levels of significance in the same pairs. Of the six anti-GAD-treated pairs the terminals of Weaver mutants were significantly larger in 4 cases than those in the wildtypes. In 2 cases no significant differences could be detected, which probably reflects the differences in the severeness of motor symptoms between individuals

Table 1. Results of the statistical procedures used to test for the significance of differences between the terminal size distributions from Weaver mutants and wildtypes (B6CBA).

	T-TEST	WILCOXON	J-V-M
Pair A	p<0.001	p<0.001	p<0.05
Pair B	p=0.065	p=0.074	p<0.05
Pair C	p=0.242	p=0.448	p=0.157
Pair D	p<0.05	p<0.005	p<0.005
Pair E	p<0.005	p<0.005	p=0.09
Pair F	p<0.005	p<0.005	p<0.005
All pairs	p<0.005	p<0.005	p<0.005

J-V-M: Johnson-Verril-Moore-test.

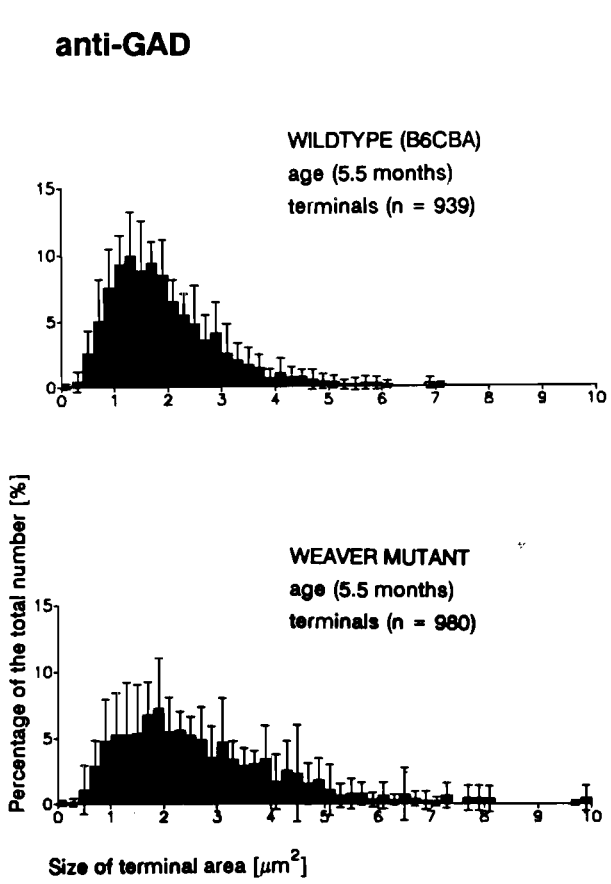

Fig. 2. Terminal size distributions of anti-GAD-treated sections from a single wildtype (B6CBA) compared to a single coprocessed Weaver mutant. The bin width of the distributions corresponds to a real cross sectional area of 0.2 µm². The level of significance is given in Table 1 (pair D).

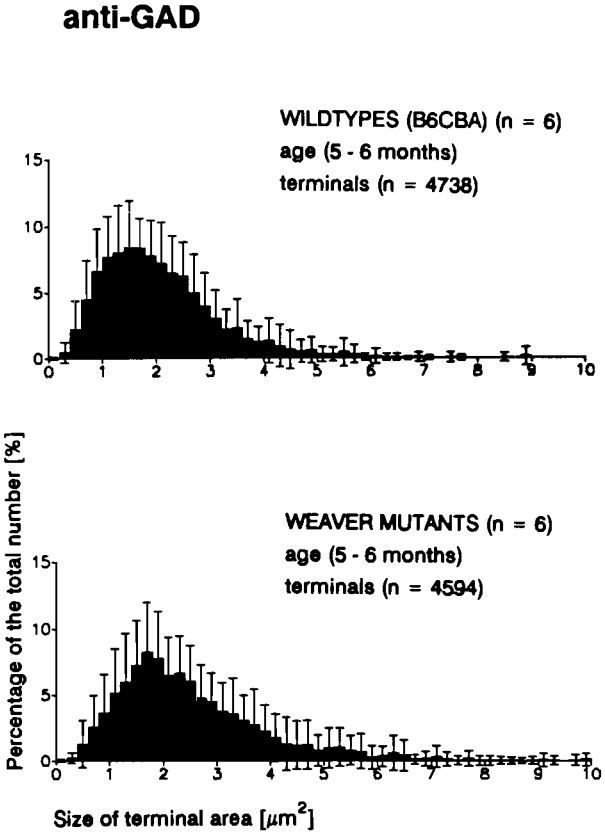

Fig. 3. Mean values of all terminal size distributions of anti-GAD-treated sections from wildtypes (B6CBA) and Weaver mutants. The bin width of the distributions corresponds to a real cross-sectional area of 0.2 µm². Terminal sizes are shifted to a larger range in the mutant compared to the control. This is especially visible when regarding the range from 0 to 1 µm² and from 4 to 7 µm². The level of significance is given in Table 1 (all pairs).

of the Weaver mutant, revealed by behavioural measurements (C. Grüsser, in preparation). Compared to controls the mean values of all pairs displayed a significant increase in terminal size in Weaver mutants.

In order to look more closely at the influence of the largest terminals, the Johnson-Verril-Moore-test, which compares only the largest 10% of the data, was also performed. Since the results of this test are not correlated to those of the other tests used, it is concluded that the differences revealed are not based on a few particularly large terminals but rather produced by the enlargement of many terminals in different size categories.

This is also visible in the size distributions in Figs. 2 and 3, where an increased number of larger terminals in the mutants, as well as a decreased number of smaller terminals are present, leading to a shift in the overall distribution towards a larger range.

Discussion

An increased size of GAD-positive terminals in the dLVN of Weaver mutants when compared to normal animals was revealed in this study by the ABC-method. This corroborates earlier findings (Bäurle et al., 1992) using GABA-antibodies and the PAP-method. For morphometric analysis, the use of GAD-antibodies instead of anti-GABA is very favourable. The maximum staining depth is dependent on antibody penetration (Sternberger et al., 1970; Hsu and Ree, 1980), but due to its larger size compared to the small GABA-molecule, the leakage of the GAD-enzyme during fixation is reduced (Hsu and Ree, 1980; Mugnaini and Oertel, 1985). Therefore, more antigen is preserved and immunopositve elements located in deeper layers of the section are visualized using the high amplification properties of the ABC-method. In contrast to the PAP-method, in which immunopositive terminals appear mainly as an edge around the somatic and dendritic surface of an innervated neuron, the quantification of individual, unsectioned and non-overlapping boutons is thus possible.

As evidenced in recent studies in Weaver using antibodies against GABA (Bäurle et al., 1992) and Calbindin D-28k (Bäurle and Grüsser-Cornehls, 1994), the vast majority of the enlarged terminals are of PC origin. The incomplete PC loss in Weaver (Blatt and Eisenman, 1985) partially deafferentiates the dLVN, and the reorganization of the cerebellar circuitry (Sotelo, 1982) caused by the absence of granule cells leads to irregular phase relationships of PC responses to vestibular stimulation (Grüsser-Cornehls, 1988). In this context, the increase in terminal size could reflect both the disorganized cerebellar output, guided through PC axons, and the reduced number of PCs, which possibly reoccupy vacated postsynaptic space by terminal enlargement. The latter possibility is uncertain in Weaver, as the number of GABAergic terminals in the dLVN is not reduced, although 2/3 of them are of PC-origin (Bäurle et al., 1992). The restored number of terminals is likely to be the consequence of collateral sprouting of which an enlargement is not inherent. Therefore, the altered electrophysiological properties (Grüsser-Cornehls, 1988) of the PC seem to be responsible for the terminal enlargement, which, therefore, could be considered as some kind of hypertrophy.

The benefit of these mechanisms in compensating for the disturbances caused by the mutation must be questioned, as the motor deficiencies displayed by Weaver mutants, which do not noticeably improve with age, are far more pronounced than in cerebellectomized Weavers or PCD-mutants (Grüsser-Cornehls, 1988; Grüsser and Grüsser-Cornehls, 1992), which completely lack PC (Mullen et al., 1976).

The reactive compensation mechanisms to the specific cerebellar histopathology found in Weaver could also be of relevance in regard to human cerebellar ataxia, for example, in the heredodegenerative disease of granule cell layer hypoplasia (Harding, 1984).

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