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Age-related development of a heterozygous phenotype in solitary neurons of the homozygous Brattleboro rat

(diabetes insipidus/immunocytochemistry/vasopressin/gene conversion)

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ABSTRACT **A single-base deletion in the single-copy vasopressin gene is the cause of diabetes insipidus in the homozygous Brattleboro rat (di/di). It results in the synthesis of an altered vasopressin precursor of which the axonal transport is blocked. Paradoxically, a small number of solitary hypothalamic neurons displays all the immunoreactivities of the wild-type vasopressin precursor (i.e., vasopressin, neurophysin, and a glycopeptide). In the present paper we provide evidence that these neurons have undergone a switch to a genuine heterozygous (di/+) phenotype; i.e., they contain the immunoreactivities of both the wild-type and the mutated vasopressin precursors. In the neural lobe, glycopeptide fibers are also present, showing that axonal transport of the wild-type precursor is restored. Moreover, the number of neurons displaying this di/+ phenotype increases markedly and in a linear way (from 0.1% up to 3% of the vasopressin cells) with age. These findings indicate that after mitotic division has ceased, genomic alterations occur in somatic neurons *in vivo*. The molecular event generating the di/+ phenotype in the di/di animal could involve a somatic intrachromosomal gene conversion between the homologous exons of the vasopressin and the related oxytocin genes.**

Since its discovery in 1961 the diabetes insipidus Brattleboro rat (phenotype, di/di) with impaired vasopressin (VP) synthesis has become an extremely useful model for many disciplines to study hypothalamic diabetes insipidus and to clarify problems related to the physiology of VP (1). The di/di rat exhibits a recessively inherited hypothalamic diabetes insipidus due to a single-base deletion in the unique VP gene (2). This point mutation results in the synthesis of an altered VP precursor whose C-terminal glycopeptide (GP) moiety, because of a frame-shift, is replaced by a nonglycosylated amino acid sequence (2). As a consequence intracellular processing and axonal transport toward the neural lobe of the mutant precursor are blocked. Paradoxically, a small number of solitary hypothalamic neurons seems to express wild-type VP gene products that are transported toward the neural lobe (3–5). To explain these results, an intrachromosomal gene conversion between the homologous exons of the VP and oxytocin (OT) genes has been proposed (6). To understand the origin of the wild-type peptides in the solitary neurons, their phenotype with respect to VP gene products was characterized by immunocytochemistry. The results show that these solitary neurons have a genuine heterozygous phenotype (termed, di/+) and that the number of these neurons increases linearly with age. The findings imply that genomic alterations occur after mitotic division has ceased and are compatible with the intrachromosomal gene conversion within the VP/OT gene locus.

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MATERIALS AND METHODS

Animals and Fixation. Male and female di/di Brattleboro rats of 18 days to 79 weeks of age were obtained from Harlan (Zeist, The Netherlands) or homebred. They were individually checked for water consumption and diuresis. The animals were anaesthetized with Nembutal (60 mg/kg, i.p.) and perfused intracardially with 0.9% NaCl (shortly) and then with 0.1 M sodium phosphate-buffered 4% (wt/vol) paraformaldehyde (pH 7.4) and pieces of tissue then were immersed overnight in the same fixation.

Immunocytochemistry. Vibratome sections (50 μm) of the hypothalamus and the neural lobe were incubated overnight with rabbit anti-rat GP antiserum (C3 final dilution, 1:1000) and GP was localized by the peroxidase-anti-peroxidase method. C3 final was raised by intracutaneous injections of the synthetic, extreme C-terminal GP sequence, residues 22–39 [GP-(22–39)], coupled to thyroglobulin (7). Subsequently, the sections were mounted on glass slides and after embedding in Entellan the number of cell profiles (subsequently referred to as cells) was determined. No staining was observed with preimmune serum or when antiserum C3 final was preadsorbed with synthetic GP-(22–39).

For the determination of the coexistence of the different parts of the VP precursor, Brattleboro rats were perfused intracardially with Bouin's fluid and hypothalamic slices were immersed overnight in the same fixative. Paraplast sections of 5 μm were collected serially on chromalum-coated slides and incubated with the following antisera: (*i*) anti-VP (126) (8) at a 1:500 dilution; (*ii*) anti-rat GP-(22–39) (C3 final) at a 1:500 dilution; (*iii*) anti-CP-14, where CP-14 is the tetradecapeptide fragment derived from the mutated region of the VP precursor (9, 10) at a 1:300 dilution; (*iv*) anti-rat neurophysin (NP; RN#4,7-19-1973) at a 1:1000 dilution (11); and (*v*) anti-rat OT–NP (PS 67) at a 1:200 dilution (12). Subsequently, the peroxidase-anti-peroxidase method was followed for sections incubated with antisera *i–iv* or a two-step method using peroxidase-labeled sheep anti-mouse IgG (13) for sections incubated with antisera *v*. Details at length on the specificity of the antisera used are presented in the references. No CP-14 immunoreactivity was present in Long–Evans rats or in di/di rats after solid-phase adsorption with CP-14.

Radioimmunoassay. VP was determined in plasma and urine as described (14).

RESULTS

By using an antiserum raised against a chemically synthesized C-terminal fragment of GP-(22–39), solitary neurons

Abbreviations: di/di, homozygous phenotype; di/+, heterozygous phenotype; VP, vasopressin; OT, oxytocin; NP, neurophysin; GP, glycopeptide; CP-14, tetradecapeptide fragment of mutant vasopressin precursor; SON, supraoptic nucleus; PVN, paraventricular nucleus; ISL, hypothalamic islands.

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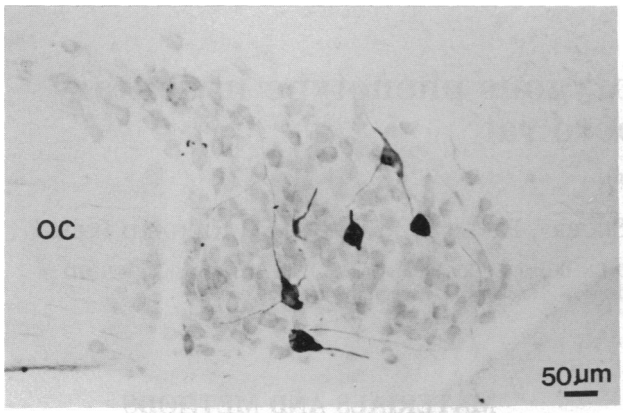

FIG. 1. GP-positive neurons in the SON of the homozygous Brattleboro rat. Intensely stained swollen cells and fibers and numerous cells with a low but consistent degree of immunoreactivity were obtained in the SON and the PVN. The latter type of immunoreactivity, which up till now has been found with all available GP antisera in the entire magnocellular component of the SON and PVN (3–5), disappeared with increasing dilutions of the antiserum (to a 1:3000 dilution), whereas the intensely stained swollen cells remained unaltered. No preference was found for a particular part of the SON or PVN. OC, optic chiasm. (Bar = 50 μm.)

were found in the supraoptic (SON) and paraventricular (PVN) nucleus of di/di Brattleboro rats (Fig. 1). These neurons were similar to those seen with antisera raised against natural GPs (3–5). The immunocytochemistry on consecutive sections demonstrated the colocalization in the solitary neurons of all wild-type VP gene products (VP, VP-associated NP and GP; Figs. 2 *a* and *b* and 3 *a* and *b*). Furthermore, it was shown that these GP-positive cells additionally still contained the immunoreactivity of the mutated portion of the VP precursor [using an antiserum against the synthetic peptide CP-14 (9, 10) Fig. 2], indicating that they had become phenotypically heterozygous. OT and OT-associated NP immunoreactivity were absent in the solitary neurons but present in a different set of neurons (Fig. 3 *c* and *d*).

The number of such GP-positive cells per total hypothalamus of male and female rats from different ages showed a marked and linear increase from 3 cells in 18-day-old rats to ≈120 cells in 79-week-old male and female rats (Table 1 and Fig. 4). This increase was found long after the magnocellular neurons have ceased to divide (i.e., fetal day 15) (16). The ratio of the solitary GP-positive cells in the SON and PVN was the same as the ratio in these nuclei of normal rats (Table 1), indicating that there is no preference for one magnocellular group over another. In neurons of the suprachiasmatic nucleus that also contain VP and GP in the wild-type rats (5), GP immunoreactivity was undetectable in all age groups of di/di animals.

As reported (3–5), GP-positive fibers were present in the neural lobe of the pituitary, especially in the older di/di rats. However, VP was undetectable in plasma and urine by

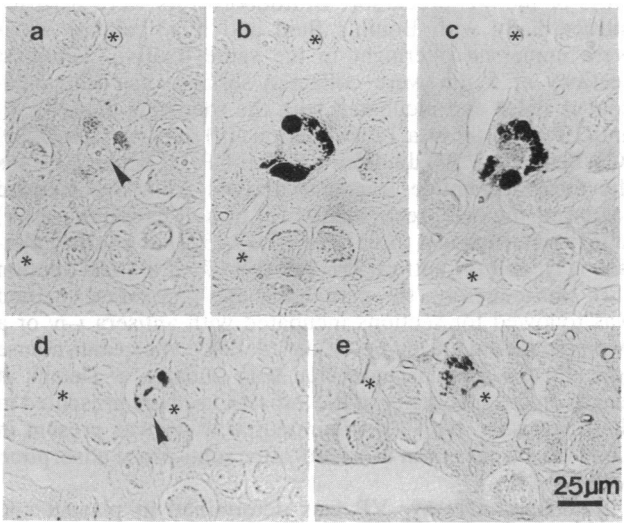

FIG. 2. +/di phenotype of magnocellular neurons of the di/di rat. Paraplast sections (5 μm) were collected serially (*a–c* and *d–e*) and incubated with the following antisera: (*a*) Anti-VP (126) (8) at a 1:500 dilution. (*b* and *d*) Anti-rat GP-(22–39) (C3 final) at a 1:500 dilution. (*c* and *e*) Anti-CP-14 (9, 10) at a 1:300 dilution. Subsequently sections were incubated with peroxidase-labeled antibodies (13). Note in *a* and *d* the nucleus and nucleolus (arrowheads) and in *a–e* the peripheral location of immunoreactivity in the Nissl substance as reported (4, 15) and present in both the intensely stained regions and part of the surrounding cells (see Fig. 1). VP immunoreactivity was only found in solitary cells. *, Capillaries present in consecutive sections. All figures have the same magnification. (Bar = 25 μm.)

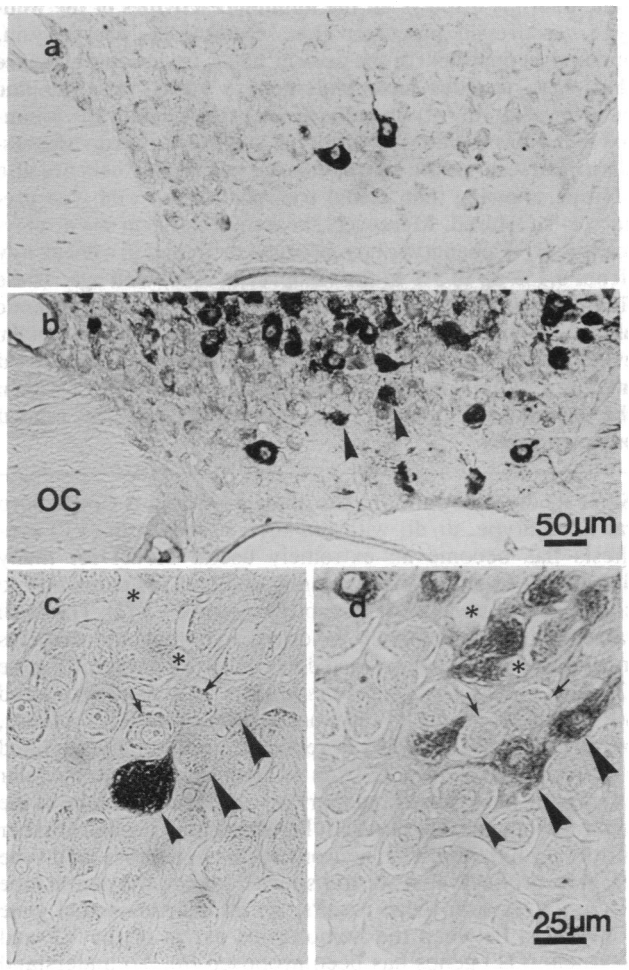

FIG. 3. VP- and OT-associated NP in solitary cells. Serial cryostat (*a* and *b*) and paraplast (*c* and *d*) sections (10 μm) of the SON were incubated with anti-GP (*a* and *c*), anti-rat NP (*b*), or anti-OT-NP (*d*). The arrowheads in *b* point to two solitary cells displaying both GP and NP immunoreactivity. In the dorsal part of the SON the OT-NP immunoreactive cells are also visible because RN#4 cross-reacts with both NPs (11). The small arrowhead in *c* and *d* points to a GP-positive, OT-NP-negative cell. In *c* the large arrowheads point to two OT-NP cells present in *d*. The arrows in *c* and *d* point to two cells out of a larger number nonreactive cells that all most probably synthesize only the mutated VP precursor. Similar to Figs. 1 and 2, most of them show a granular staining in their periphery with GP antiserum (*c*). *, Capillaries present in consecutive sections; OC, optic chiasm.

Table 1. Mean number of GP-immunoreactive cell profiles in homozygous Brattleboro rats as determined in 50-μm-thick Vibratome sections

Age, weeks	Location of section	GP-immunoreactive cell profiles, no. Male	Female
2.6	SON	2.9 ± 0.8 (7)	1.7 ± 0.7 (7)
	PVN	0.4 ± 0.3	1.3 ± 0.4
	ISL	0.3 ± 0.3	0.0 ± 0.0
	Total	3.6 ± 1.1	3.0 ± 0.7
9	SON	12.4 ± 1.2 (5)	9.0 ± 1.4 (7)
	PVN	6.8 ± 1.5	2.6 ± 0.6
	ISL	1.6 ± 0.7	1.1 ± 0.5
	Total	20.8 ± 0.4	12.7 ± 1.2
36	SON	34.2 ± 2.1 (5)	34.2 ± 6.1 (6)
	PVN	9.2 ± 2.1	13.6 ± 1.6
	ISL	4.4 ± 0.9	6.0 ± 1.2
	Total	47.8 ± 2.2	53.8 ± 6.7
48	SON	42.0 ± 11.5 (4)	53.4 ± 3.8 (5)
	PVN	16.0 ± 5.2	12.2 ± 1.7
	ISL	12.5 ± 1.2	6.4 ± 1.7
	Total	70.5 ± 13.3	72.0 ± 4.0
60	SON	68.0 ± 2.4 (5)	61.0 ± 7.5 (5)
	PVN	18.6 ± 2.5	18.6 ± 1.5
	ISL	6.8 ± 1.5	9.6 ± 1.8
	Total	93.4 ± 2.9	89.2 ± 8.9
79	SON	98.8 ± 8.0 (5)	84.4 ± 7.2 (5)
	PVN	19.6 ± 3.3	17.0 ± 3.2
	ISL	8.6 ± 1.3	12.2 ± 2.0
	Total	127.0 ± 6.3	113.6 ± 7.6

Results are mean ± SEM; numbers in parentheses are *n*. ISL, hypothalamic islands. The SON/PVN cell ratio of all groups is 3.7 ± 0.5 (mean ± SEM); Swaab *et al.* (8) reported a ratio of 3.2.

radioimmunoassay (14) (<1.3 pg/ml) of di/di animals of 79 weeks of age and no recovery from diabetes insipidus was noted.

DISCUSSION

To account for GP-positive neurons in di/di rats, Ivell (6) has put forward the hypothesis that somatic gene conversion may occur between the OT gene and mutated VP gene. The highly homologous exons B of the OT and mutated VP genes could interact since they are closely situated (17). The deleted VP gene would be changed, by mismatch, repair, or actual strand exchange involving exon B (Fig. 5). This genetic alteration would then lead to restoration of the reading frame and GP synthesis. This would be a very infrequent intrachromosomal event and would, therefore, statistically affect only one of the VP alleles in any one cell. Consequently, the GP-positive cells would be few in number and have a heterozygous phenotype. Furthermore, the enzyme systems involved are probably similar to those of the DNA repair system and are likely to be independent of mitotic cell division or meiosis.

The immunohistochemical results support this hypothetical model by demonstrating the age-related development of

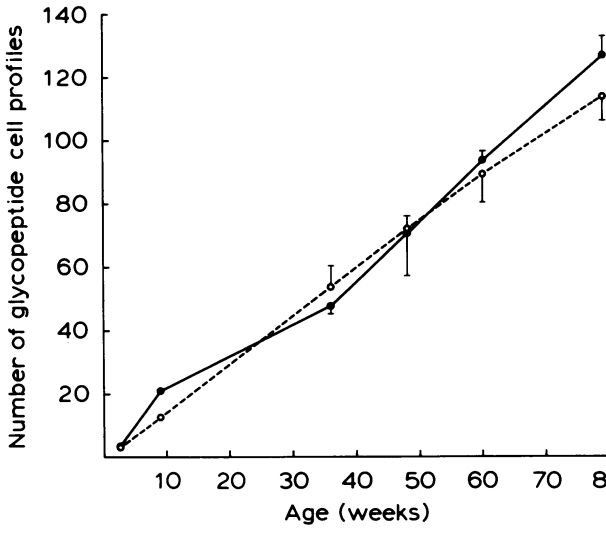

FIG. 4. Linear relationship between the total number of GP-immunoreactive profiles in the hypothalamus of male (●) and female (○) homozygous Brattleboro (di/di) rats and the age in weeks.

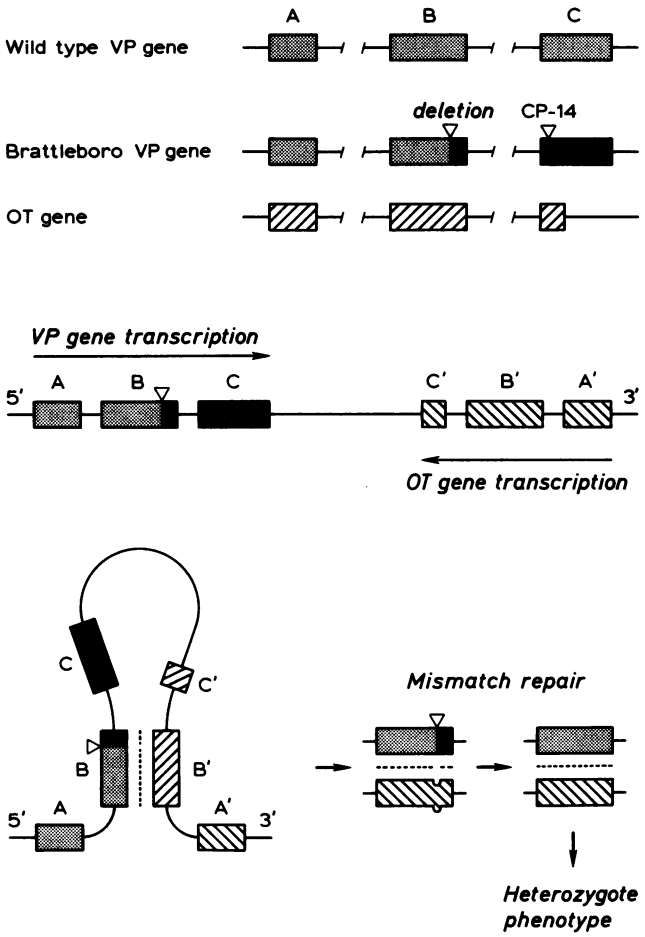

FIG. 5. Proposed model of the phenotypic change in the di/di rat. The VP and OT genes of both the wild-type and di/di rats are located in the same locus and each consists of three exons (A, B, and C) separated from each other by two introns. In the NP-encoding region of exon B, a guanosine residue is missing in the VP gene of the di/di rats, which leads to a shift in the reading frame and a different C terminus of the VP precursor [this includes a 14-amino acid peptide, CP-14, which was chemically synthesized and used to raise the antiserum against the mutated VP precursor (2, 9, 10) (Fig. 2)]. In a part of the NP-encoding regions (exon B) of the VP and OT genes, there is a stretch of 198 nucleotides with a homology of ≈97% (18). The di VP gene is deleted in this homologous region (2). In the rat the VP and OT genes are only separated by ≈11 kilobases and in inverted orientation [i.e., transcribed from opposite DNA strands (17), A', B', C': noncoding regions of OT gene]. Therefore, a hairpin loop can be formed between the nonallelic homologous regions of the two genes present on the same DNA strand. The homologous OT-NP exon could then act as template for repair of the single mismatch, using nuclear enzymes. Whether the repair involves nucleotide excision and replacement or actual strand exchange (6) is not pertinent to the phenotypic consequences of this gene conversion. Since statistically in any one cell, one chromosome of the allelic pair is ever likely to be repaired, the phenotype in those few cells undergoing correction will always be heterozygous and would, therefore, express both GP and CP-14 immunoreactivities.

neurons with the di/+ phenotype in the homozygous di/di rat. The age-dependent increase of the number of GP cells most probably explains the difference in the number of these cells reported in the literature (4, 5). The absence of GP-positive cells in the suprachiasmatic nucleus need not disagree with the proposed model of gene conversion, since the latter event probably depends on the physical status of the DNA, particularly in regard to chromatin structure: levels of DNA repair have been linked to transcriptional activity (19). The magnocellular neurons of the SON, PVN, and ISL in the di/di rat are under chronic hyperosmotic stress. They are indeed hypertrophic (e.g., nucleolar size; ref. 20) and transcriptionally very active (21–24). In contrast, the VP neurons of the suprachiasmatic nucleus are insensitive to hyperosmolality (25). The severe osmotic stress in the di/di rat would also be expected to cause a hypertrophy of the few newly generated GP- and VP-positive cells (see Fig. 1). Consequently, the ultrastructural appearance of these cells in the di/di rat could be different from the VP neurons of the di/+ heterozygous Brattleboro rat (15, 20), which suffers only slightly from hyperosmolality (1) and whose neurons will have had a normal functional ontogeny with appropriate afferent innervation (26).

In eukaryotes, genomic DNA can be altered by a number of naturally occurring processes including deletion, insertion, gene conversion, and recombination. The occurrence of these processes during meiosis in the germ-line genome has been recognized in cells expressing globin and the major histocompatibility antigen genes (27, 28). Immunoglobulin genes display somatic recombination before proliferation (29). Our results suggest the existence of a somatic, post-mitotic, nontumorigenic, genetic alteration occurring *in vivo* in magnocellular VP neurons of the di/di Brattleboro rat. By this alteration an inborn error is partly repaired and as a consequence normal post-translational processing and axonal transport of wild-type gene products are restored. The age-related development of the heterozygous di/+ phenotype within a homozygous di/di animal could well involve a somatic intrachromosomal gene conversion promoted by the close juxtaposition and deletion, which shortens the distance between the VP and OT genes (30) and high homology of the VP and OT genes. Alternative mechanisms, which could result in the restoration of a wild-type VP precursor (e.g., alternative splicing in the VP gene or expression of a second VP gene), are not likely in view of the extensive knowledge of the molecular biology of the VP gene (6). Direct evidence for the molecular events underlying this switch in phenotype requires elucidation of the nucleotide sequence of the revertant VP mRNA in the solitary GP-positive neurons. The paucity of such cells will make this still a very difficult task.

In conclusion, it is possible that post-mitotic phenotypic changes, comparable to those demonstrated in the present study, may also occur in other gene families with highly homologous domains. The Brattleboro rat, due to the clear contrast between the di/di and di/+ phenotypes, is an excellent model to study the phenotypic switch *in vivo* in certain neurosecretory neurons.

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